United States
Department of
Agriculture

1995 Progress Report of Food Safety Research

Conducted by ARS

EXECUTIVE SUMMARY

This report documents the ARS research progress in 1995 to assure the safety of food products of animal origin. Highlights of the achievements are outlined below. These are further documented in the report along with many others.

Food safety starts with a healthy animal also free of organisms which are pathogenic for humans. Automatic aerosal application in spray cabinets of a characterized competitive exclusion culture protected newly hatched chicks against Salmonella colonization equally as well as direct application of the culture by crop gavage. This new application method at the hatchery can economically treat large numbers of commercially hatched chicks. Exclusive licensing for commercial production of the culture has been completed through a CRADA, and the licensee is producing the amounts for the final testing required by the FDA.

Ten monoclonal antibodies against E. coli 0157:H7 antigens have been produced and used to develop assays for E. coli 0157:H7 detection. One assay format was found suitable for the mass screening for E. coli needed in a clinical or diagnostic laboratory. The ten antigens were also formatted into a simple and rapid test suitable for individual clinical or food samples or for working environments such as slaughter plants. This latter test has been developed in collaboration with a commercial partner and is in the final stages of performance evaluation.

Rapid and sensitive multiplex PCR methods were developed which can detect both Campylobacter coli and C. jejuni and differentiate between the two species. Conventional methods to detect and to identify Campylobacter involve lengthy incubations and specialized gas mixtures. The assays can also rapidly differentiate Arcobacter from other fastidious, Campylobacter-like organisms which may be found in swine. This technology is being transferred to the FSIS.

ARS research is developing methods to control Cryptosporidiosis, a human and animal pathogen which may contaminate drinking water. Genetic material from Cryptosporidia resulted in a detectable immune response in calves, pointing the way to a useful vaccine. The infectious stage of Cryptosporidia could be inactivated in 1 minute at 72.4° C.; however, exposure of Cryptosporidia to concentrated laundry bleach did not inactivate the parasite, as would be expected with bacteria.

To control pathogens in the **slaughter and processing area**, ARS scientists demonstrated that chlorine dioxide achieved acceptable bacterial reductions using four to five times less chlorine than required when the usual gaseous chlorine product was used. These studies provided the essential research information to enable industry to obtain approval from the Food and Drug Administration for the use of chlorine dioxide in poultry chiller water.

To more effectively clean beef carcassses ARS used a commercial steam-vacuum system in conjunction with a commercial combination wash of hot water (72° C) at low pressure (20 psi) combined with a high pressure (125 psi) warm water (30° C) wash. This combination treatment reduced several measures of bacterial levels on the contaminated beef carcasses by 3 to 4 logs, and demonstrates that the treatment system could reduce the amount of trimming needed on beef carcass processing lines.

A near-infrared system with the probe in a housing, was shown to be able to differentiate cadaver and septicemic broilers from normal birds in poultry slaughter and processing. Because the sensing and computer classification times are very rapid, plans can now be made for integrating the system on line in a broiler slaughter and processor plant for studies in a real time situation.

Post slaughter pathogen control comprises those studies addressing problems of product processing following the initial chilling of carcasses. A firefly luciferase gene on a plasmid vector was introduced into three strains of E. coli 0157:H7 making them highly luminescent, but otherwise indistinguishable from their parent strains in standard microbioogical tests. These bioluminescent strains could be useful as positive controls in microbiological assays, and in studies of bacterial injury and recovery and the effects of antibacterial agents. The FSIS is evaluating them for use in their regulatory program.

Growth kinetics were modeled in a media system of a three strain mixture of Clostridium perfringes, a common food pathogen. The effects and interaction of temperature, pH, sodium chloride, sodium pyrophosphate were assessed. The data indicate that sodium pyrophosphate can have significant bacteriostatic activity against C. perfringes and may provide processed meats with protection against this pathogen, particularly if employed in conjunction with a combination of acidic pH, high salt concentration and adequate refrigeration.

Studies to identify the mechanism by which irradiated pathogens become more sensitive to heat than are non-irradiated cells utilized measurements of both DNA damage in vivo and the integrity of the cytoplasmic membrane. Heating followed by irradiation was additive. However, when irradiation preceded heating in the absence of oxygen, more bacterial cells were inactivated, and the irradiation damaged DNA was more sensitive to heat than would be expected from the sum of the two processes. This improved understanding of microbial susceptibility can lead to more effective food processing.

Residue detection focuses on drugs, environmental contaminants, and process generated contaminants. Methodology to determine melengestrol acetate residues in meat was developed which uses a combination of supercritical fluid extraction (SFE) and solid phase extraction(SPE) techniques to produce an extract suitable for analysis. This SFE-SPE procedure utilizes a total of 12 ml of organic solvent per fat tissue sample compared with greater than 1.9 liters consumed in the current extraction procedure. The method is being transferred to the FSIS for their evaluation and implementation.

The FDA has approved ceftiofur, a veterinary cephalosporin antibiotic for use in food producing animals. A monoclonal antibody utilizing a protein conjugate of the ceftiofur metabolite as the immunogen was developed and formatted into an ELISA assay. Preliminary experiments indicate that ceftiofur can be detected in milk samples without the need for any extraction or cleanup steps; and studies have been initiated to detect ceftiofur in beef.

Catalase activity in ground beef and pork was determined in model system samples cooked from 60 to 71.1° C. Analysis of the results of various time-temperature combinations indicated that the method is suitable for use by the FSIS laboratories, and state and local health departments to verify compliance with beef and sausage pattie product regulations. Further, the method could be used in fast food quality assurance programs to assure compliance with the regulations.

ANNUAL REPORT ON FOOD SAFETY RESEARCH CONDUCTED BY ARS

1995

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CONTROL SALMONELLA IN DOMESTIC ANIMALS

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OBJECTIVE A: To develop a cost effective means to prevent and/or control salmonellosis in poultry.

PROGRESS A: Research efforts were focused on further development of the patented characterized competitive exclusion culture to control Salmonella colonization in broiler chickens. The characterized culture composed of 29 strains of cecal bacteria was tested for efficacy at various dosage levels under different spray application conditions. Automatic application of the culture in spray cabinets provided protection against Salmonella colonization in newly hatched chicks that was equal to the protection provided by direct application of the culture by crop gavage. The application of the culture in automated spray cabinets at the hatchery will provide the opportunity to treat large numbers of commercially hatched chicks shortly after hatching.

The results of dosage titration studies indicated that chicks sprayed with 0.25 ml of the culture containing 10^7 or 10^8 anaerobic colony forming units were significantly ($P<.01$) more resistant to Salmonella colonization than were nontreated controls. Additionally, spray application of chicks with 10^6 or 10^7 anaerobic colony forming units of the culture significantly ($P<.01$) decreased the spread of Salmonella from infected chicks to noninfected pen mates by 75 to 100%.

Lyophilized seed stock of the original characterized culture that had been maintained frozen for 2 years was used to inoculate and start a new continuous-flow culture. The new culture provided protection against Salmonella colonization in chicks that was equal to the protection provided by culture which had been maintained in continuous culture for the same 2 year time period. The results indicated that a lyophilized frozen seed stock may be utilized as inocula to generate new continuous-flow cultures and perpetuate the continued maintenance of the characterized culture.

Investigations to further define the mechanism(s) of increased resistance to Salmonella enteritidis (SE) organ infectivity in chickens conferred by the prophylactic administration of SE-immune lymphokines have continued. It was demonstrated that a lymphokine-mediated heterophilic inflammatory response significantly increases the innate resistance of chickens against organ infectivity by SE. The inflammatory response is characterized by: (a) a significant granulopoiesis and increase in circulating heterophils within 4 h, (b) the potentiation of the biological activities of the heterophils including adherence, chemotaxis, phagocytosis, and bacterial killing capacity, (c) up-regulation of CD11b/CD18 surface markers on heterophil membranes which are responsible for specific receptor- mediated in vitro adherence, and (d) the directed influx of these activated heterophils to the site of bacterial invasion. We further described that the injection of ILK and live, but not formalin-killed, SE resulted in an increased influx of inflammatory heterophils into the peritoneal cavity that peaked at 4 h after the injections with no increase in peritoneal macrophages. The heterophil accumulation was not influenced by polymyxin B nor exogenous LPS, but was sensitive to heat treatment of the ILK. Treatment of the chicks with inhibitors of arachidonic acid metabolites did not abrogate

the induced heterophil accumulation. Furthermore, we found that the chemotactic cytokine, IL-8, is produced in the local environment by the host in response to both the SE infection and the ILK. It was further established that IL-8 was a major chemotactic factor mediating the ILK/SE- induced recruitment of heterophils to the site of SE invasion. The presence of a colony stimulating factor in the ILK which preferentially supported the in vitro growth of granulocytic bone marrow colonies and caused the emigration of bone-marrow derived granulocytes into the peripheral blood within 4 hours after injection of ILK in vivo was also described. Additional experiments found that ILK produced from either SE-immune chickens or turkeys reduced SE organ invasion in both day-old chicks and turkeys. Recent studies have demonstrated that the prophylactic administration of ILK induced from SE-immune lymphocytes conferred protection to neonatal broiler chicks against mortality and organ invasion by the casuative agent of avian typhoid, S. gallinarum.

IMPACT/TECH TRANSFER: Exclusive licensing for commercial production of the patented culture was completed through a CRADA with Milk Specialties Co., Dundee, IL. The Bioscience Division of Milk Specialties is presently maintaining the culture in scaled up commercial size continuous-flow culture systems. A CRADA has been established with Embrex on the lymphokine project and they are applying for a license to our patent (application submitted). Additionally, a second patent application is being made. The research indicates these lymphokines are important to the control of invasive Salmonella.

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Fan, Y. Y., Ricke, S. C., Scanlan, C. M., Nisbet, D. J., Vargas-Moskola, A., Corrier, D. E. and DeLoach, J. R. 1995. Utilization of a differential rumen fluid based carbohydrate agar media for culturing selected cecal bacteria from chickens. J. Food Prot. 58:361-367.

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Ha, S. D., Nisbet, D. J., Corrier, D. E., DeLoach, and Ricke, S. E. 1995. Comparison of Salmonella typhimurium and selected chicken cecal bacteria survivability after specific amino acid-limited batch growth. J. Food Prot. (Accepted)

Ziprin, R. L., Kogut, M. H., McGruder, E. D., Hargis, B. M. and DeLoach, J. R.1995. Efficacy of Salmonella enteritidis-immune lymphokines from chickens and turkeys on SE organ invasion in day-old chicks and turkey poults. Avian Dis. (Accepted).

Kogut, M. H., McGruder, E. D., Hargis, B. M., Corrier, D. E. and DeLoach, J. R. 1995. Role for IL-8 in the accumulation of peritoneal heterophils in chicks following the intraperitoneal administration of Salmonella enteritidis-immune lymphokines and Salmonella enteritidis. Scand. J. Immunol. (Accepted).

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PATHOGENESIS OF SALMONELLA ENTERITIDIS IN CHICKENS

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OBJECTIVE A: Develop an epidemiological model that describes the dissemination of Salmonella enteritidis in animal populations.

PROGRESS A: We have identified, in collaboration with APHIS scientists, that culturing of spleens obtained from mice caught in chicken houses is a more reliable predictor of the presence of egg-contaminating S. enteritidis than is environmental sampling. Also, culture of organs is more likely to detect S. enteritidis in a house regardless if contaminated eggs are detected. During these studies, one phenotype was found that correlated with microbial differentiation and production of virulence factors. Extraction of outer membrane lipopolysaccharide (LPS) from this phenotype, which characteristically hyperflagellated and migrated across 2% inhibitory agar surfaces, indicated that there was a preponderance of one LPS structure present in the outer membrane. These results were used to develop an epidemiological model, which suggests that salmonellosis might be composed of four distinct phases in animal populations as monitored by analysis of LPS structure. This model was evaluated in experimentally infected chicks using LPS characterized variants for infection. Results indicate that Koch's postulates were fulfilled and that the model is valid for describing the pattern of infection of Salmonella enteritidis in animal populations.

IMPACT/TECH TRANSFER: Epidemiological modeling suggests that while European countries such as England and Germany are most likely in the third, or worst, phase of affliction, the U. S. is probably only in the second phase. It is of note that European countries trace illness from S. enteritidis to both meat and eggs, so it is conceivable that additional cases of S. enteritidis attributable to meat contamination might begin to occur in reportable numbers if the United States progresses into the third phase. The model also predicts that there could be a lag between infection phases, which suggests that a reduction could precede an expansion of S. enteritidis in mouse and chick populations.

OBJECTIVE B: Assess whether phage type 4 Salmonella enteritidis (SE) strains represent a more serious threat to public health and to poultry than strains of phage types more commonly found in the U.S.

PROGRESS B: We determined that some, but not all, phage type 4 S. enteritidis strains were more virulent for chicks than were phage types found more often in U.S. poultry. S. enteritidis contamination of eggs has been an important public health problem in the U.S. for several years.

Phage type 4 (PT4) S. enteritidis, which until recently had not been isolated from poultry in the U.S., has been associated with reports of clinical disease in British poultry flocks. The detection of PT4 S. enteritidis in poultry in the U.S. has necessitated an evaluation of the risk posed by this phage type to determine whether revision of the current S. enteritidis control strategy is necessary. We assessed the ability of PT4 isolates to cause severe illness in both egg-type and meat-type chicks in comparison to isolates of phage types more commonly found in U.S. poultry. The average level of severe illness in chicks associated with PT4 S. enteritidis isolates (including some recent isolates from U.S. laying

flocks) was greater than that of SE isolates of other phage types but less than that of S. pullorum, a serotype historically associated with significant disease losses in poultry. Significant differences in virulence, however, were observed within the set of PT4 strains tested. The incidence of severe illness following S. enteritidis infection was higher in egg-type chicks than in meat-type chicks.

IMPACT/TECH TRANSFER: This demonstrated to regulatory officials and the poultry industry that PT4 S. enteritidis strains can sometimes be more virulent for chicks than other phage types, but this generalization does not apply to all PT4 strains.

OBJECTIVE C: Evaluate the role of immunization of hens with killed Salmonella enteritidis vaccines in reducing human food-borne outbreaks.

PROGRESS C: Epidemiological studies of human food-borne S. enteritidis outbreaks where eggs or egg products were implicated as the potential source indicated that egg abuse (pooling large number of egg contents and allowing these eggs to sit at ambient temperatures for long periods) may have been responsible. Such abuses could result in expansion of the low numbers of S. enteritidis normally deposited in eggs to high numbers which would more readily cause human infection. Serum antibodies accumulate in yolk during egg formation and the yolk antibody repertoire mirrors that found in serum. A study was conducted to examine if anti-S. enteritidis antibodies found in egg yolks following vaccination with an S. enteritidis bacterin will affect the in vitro growth of 10 S. enteritidis seeded into pooled egg contents (mimicking the abuse of the egg in eating establishments). The S. enteritidis grew well in pooled egg contents from control hens 80-100% of the pools were SE positive following 24 hour incubation and grew to densities greater than 100 million/ml. Significantly fewer egg pools from vaccinated hens were positive following this same incubation period and the levels achieved in these pools were less than 10,000. The inhibition could be observed in immune egg pools diluted 1:5 in control eggs and, on occasion, in pools receiving a 10-fold higher inoculum. Previous studies had shown that vaccinating hens with an S. enteritidis bacterin reduced the incidence of contaminated eggs.

IMPACT/TECH TRANSFER: The current results indicate that vaccination may have the added benefit of inhibiting the growth of S. enteritidis, at least for a short time, in those eggs still contaminated and serve to further protect the U.S. egg consumer.

OBJECTIVE D: Develop virulence assays that detect organ-invasive, egg-contaminating Salmonella enteritidis.

PROGRESS D: We have identified that the virulence potential of S. enteritidis of any phage type is assayable by characterization of outer membrane complex carbohydrates. Results indicate that log scale increases in infectivity as measured by recovery of organisms from chick spleens occur when a particular structure of LPS associated with cell migration is produced. This structure lacks a single-sugar modification in the core region and has a marked increase in the ratio of O-antigen to core sugars. These results also indicate that phage typing does not predict virulence potential for any one isolate except for those phage types lacking O-antigen (i.e. pt 23). which are avirulent. Phage types with O- antigen examined to date (i.e. 4, 8, 13A, 14b) exhibit significant variation in virulence that correlates with variations in complex carbohydrates on the outer membrane. Proteus mirabilis, which is a pathogenic organism that cyclically produces well-characterized virulence factors, was analyzed using genetics in addition to carbohydrate characterization. Results indicate that the absence of the predominant carbohydrate that facilitates cell migration for this organism ablates virulence potential

even when all other proteinaceous virulence factors are transcribed. Thus, at the genetic level, results indicate that virulence potential of environmental pathogens cannot be assessed without evaluating the carbohydrates on the outer membrane of bacteria.

IMPACT/TECH TRANSFER: These findings impact other scientists in industry and research who perform vaccine efficacy trials since results indicate that carbohydrate characterization of S. enteritidis is more important than phage type for predicting virulence. A CRADA has been established to evaluate the efficacy of vaccines against highly virulent strains of S. enteritidis.

OBJECTIVE E: Develop and evaluate sensitive and efficient methods for testing chickens and eggs for Salmonella enteritidis.

PROGRESS E: We identified differences between S. enteritidis strains in the ability to multiply in egg contents that can affect the probability of detecting contamination by culturing. Culturing of eggs to detect S. enteritidis has become a vital component in programs to detect laying flocks that potentially threaten public health. Culturing pools of egg contents for S. enteritidis usually depends on an initial period of incubation of the pools to allow small initial bacterial numbers to multiply to levels more likely to be detected. Any differences between S. enteritidis strains in their abilities to multiply in egg pools during incubation could ultimately affect the likelihood of detection by culturing. We assessed whether 12 S. enteritidis strains (of various phage types) were able to grow to reach different final levels during a 24-hour incubation period in egg contents pools. Some S. enteritidis strains grew to levels that were more than a thousand times greater than other strains. However, when the egg pools were supplemented with iron during incubation, S. enteritidis multiplication was increased significantly and the differences in growth between strains were less evident.

IMPACT/TECH TRANSFER: This demonstrated to regulatory officials and diagnostic laboratories that culture methods for SE in eggs must account for the possibility that some strains may grow rather slowly in egg pools if these methods are to ensure that any possible S. enteritidis organisms are detected.

OBJECTIVE F: Evaluate rapid methods for detecting Salmonella enteritidis in pooled egg samples

PROGRESS F: Culturing eggs for contamination remains a primary component of programs to reduce or control S. enteritidis in laying flocks. The method of choice is the incubation of the egg contents for 72 hours at ambient temperature and then direct plating the egg contents onto differential media. The time required for a final identification of S. enteritidis contaminated egg samples is 96 to 120 hours. Studies were conducted to shorten the time from initiation of egg culture to receipt of the culture result. Incubating the pools at 37 °C was found to shorten the culture time to 24 hours. To further reduce the time required to identify contaminated egg samples, we developed a detection assay which utilized magnetic beads having anti-Salmonella antibodies affixed to their surface to bind the S. enteritidis. The bead-S. enteritidis mixture could be pulled out of the egg contents using a magnet and the S. enteritidis could then be detected via an ELISA utilizing a horse radish peroxidase-conjugated monoclonal anti-S. enteritidis flagella antibody as the indicator system. The ELISA detection capability was as sensitive as direct plating and the assay could detect S. enteritidis 24 hours sooner than the plating protocol. Further, if the beads were plated after the ELISA was completed, several more positive pools were detected which were missed by the ELISA and direct plating.

IMPACT/TECH TRANSFER: Shortened detection times coupled with the potential for automating the assay could increase the potential utility of the assay system for future industry or federal quality assurance programs. A CRADA has been established with the company that manufactures the magnetic beads to develop protocols for enhancing the sensitivity of the ELISA and adapting the assay to a microtiter format.

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CONTROL OF SALMONELLA IN DOMESTIC ANIMALS

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OBJECTIVE A: Develop control procedures to prevent infection of eggs and chicks by salmonellae in hatcheries including treatment of equipment, eggs, chicks, and/or breeder flocks with chemicals.

PROGRESS A: An automated spray washer was shown to effectively sanitize fertile hatching eggs before placement in hatchery setting cabinets. Polyhexamethylenebiguanide hydrochloride and hydrogen peroxide were the most effective sanitizer tested. The use of a spray washer is easier and more compatible to commercial operations than earlier tested dip methods. These data show that effective washing and sanitizing of hatching eggs at the breeder farm can eliminate or reduce bacteria including salmonellae that enter the hatchery. To determine microbial levels in hatching cabinets a comparison was made between an automated air sampler and open plates. The automated air sampling machine was shown to be more efficient for enumerating Enterobacteriaceae while the open plate enrichment system detected more salmonellae. A survey comparing salmonellae contamination rates was completed in the same commercial hatcheries as in 1988. The 1995 contamination rates were 22.5, 52.2 and 12.8% in hatcheries A, B and C, respectively compared to the 1988 contamination rates of 75.5, 90.9 and 66.7% in the same hatcheries. **IMPACT/ TECH TRANSFER:** These data indicate that the hatching industry has made changes as a result of our 1988 survey that have significantly reduced the levels of salmonellae entering the grow-out farm from the hatchery.

OBJECTIVE B: Develop control procedures to prevent colonization of chickens and/or turkeys with competitive exclusion cultures.

PROGRESS B: Our mucosal competitive exclusion cultures (MCE) patent was issued by the U.S. patent office and has been licensed for commercial distribution by the Continental Grain Company. MCE was tested in a 25,000 thousand turkey poult field trial. After 5 weeks in the brood house, salmonellae were detected in 3% of cecal samples from MCE treated poults compared to 47% of untreated poults. This difference in colonization did not carry over after transfer of poults to final grow-out houses. We are investigating whether the stress of moving the poults disrupted gut microflora and leads to higher levels of salmonellae. MCE and other strategies will be tested to reduce spread of salmonellae at this apparent critical control point. When the yeast, Saccharomyces cerevisiae var. boulardii (Sb) when added to feed, Salmonella colonization was reduced from about 65% in controls to about 15% in treated chicks. Work is continuing to determine the most appropriate means and routes of yeast administration to chickens for reducing Salmonella populations associated with the birds. **IMPACT/TECH TRANSFER:** A patent covering the feeding of Sb and other yeast to chicken has been applied for and a patent pending number issued.

OBJECTIVE C: Determine the effect of different cold storage temperatures on the microbiological condition of processed chicken.

PROGRESS C: In response to a high priority FSIS research request the microbiological profile of chickens were determined after storage at 0, 10, 26, 32 and 40 °F. Salmonellae

did not change and coliform bacteria counts declined at all storage temperatures. After 7 days storage, mesophilic bacteria increased by 2 Logs at 40 °F. Psychrophilic spoilage bacteria increased by 3, 1.5 and 1.0 Logs at 40, 32, and 26 °F, respectively. At all other temperatures and storage conditions, bacteria counts remained the same or declined. **IMPACT/TECH TRANSFER:** This data was useful to FSIS in helping rewrite its new frozen poultry regulation.

OBJECTIVE D: Determine which method or methods of shipping poultry samples will maximize the detection of Salmonella.

PROGRESS D: Maximum recovery of salmonellae from processed broiler carcasses was obtained when rinse fluid and chiller water sample were ice packed and not frozen before transport to the laboratory. **IMPACT/TECH TRANSFER:** These data allow government, industry and university researchers and regulators to more accurately know the salmonellae status of field samples which are collected and sent to central laboratories.

OBJECTIVE E: To determine a rapid method to detect temperature abuse of fresh broiler chickens.

PROGRESS E: It was determined that monitoring Escherichia coli as an indicator of temperature abuse was more effective than monitoring mesophilic bacterial populations. CM coliform medium with 2% added dextrose was shown to be the ideal medium to enumerate E. coli in broiler carcasses. **IMPACT/TECH TRANSFER:** The use of this optimized medium in an automated impedance system offers the poultry industry a viable, reliable and rapid method to determine if processed chicken had been previously temperature abused.

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PATHOGENESIS, TRANSMISSION, AND CONTROL OF SALMONELLOSIS IN SWINE

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OBJECTIVE A: To identify virulence factors important in the pathogenesis of Salmonella choleraesuis and Salmonella typhimurium in swine

PROGRESS A: Identification of antigens which may be important in eliciting an immune response: We produced soluble antigen and lipopolysaccharide (LPS) antigen extracts for the development of indirect ELISA assays which may be used to monitor the immune response and identify carrier animals. A mixed ELISA format was developed for both antigen preparations. Antigens were prepared from 4 serogroups prior to coating the ELISA plates in order that the immune response to Salmonella within the major serogroups recovered from swine may be monitored. The use of the 2 antigen preparations gives different results in that LPS produces a more sustained immune response which is indicative of exposure but may not necessarily identify if the animal is a carrier. Use of the soluble extract indicates that the immune response is shorter in duration which may be more useful in detecting carrier animals.

PLAN: Test combined use of both antigens in a HACCP monitoring program to enhance the probability of detecting those animals which have been exposed to the antigen versus those that may be true carriers.

IMPACT/TECH TRANSFER: Information regarding detection of carrier animals will benefit those seeking to implement HACCP plans. This work has been presented to commodity groups and other professionals at scientific meetings.

OBJECTIVE B: To define the epidemiology and transmission of Salmonella in swine

PROGRESS B: Survival and transmission of Salmonella choleraesuis: Feces was collected from swine infected with Salmonella choleraesuis and was stored in either a dried or wet form. Survival was monitored by both qualitative and quantitative bacteriology. Results indicate that Salmonella choleraesuis survives in both states. However, duration of survival is significantly longer in feces that has been dried as opposed to being maintained in a wet environment. Additionally, time of collection after shedding also appears to affect survival. Salmonella choleraesuis in feces collected and dried after 7-10 days from infected swine survives for a longer period of time than S. choleraesuis in feces collected within 4 days following infection. Challenge of swine by intranasal inoculation with dried feces did not affect the virulence, clinical outcome or tissue distribution of the Salmonella infection. These data indicate that appropriate sanitation measures are critical when designing control plans in swine units and that any dried organic matter may serve as a reservoir for Salmonella.

Salmonella spp. in feed and feed ingredients: We have conducted a survey of feed and feed ingredients to determine the prevalence of Salmonella spp. Feed was collected from 23 farms in 8 states. Results indicate that Salmonella was isolated from at least one feed or ingredient on 11 (48%) of the farms. Of the 950 samples taken, 29 (3.1%) were positive

for Salmonella. Ten different serotypes have been identified. Salmonella isolated from samples taken from feeders, troughs, floor or bulk storage areas could have originated from sources other than the feed or ingredients. Salmonella isolated from the bagged feed ingredients, fishmeal and grower-finisher premix probably originated from those components. These data indicate that Salmonella may be a common inhabitant of the environment in which swine are raised.

Salmonella spp. from feed transport trucks: We have conducted a survey of Salmonella in feed transport trucks. Twenty-five culture swabs were taken from 22 trucks from 3 states; feed samples were also cultured from 17/22 trucks. All swabs were cultured for Salmonella spp. Results indicate that 5/22 (22.7%) of the trucks were positive for Salmonella spp. Salmonella was isolated from approximately 4/540 swabs (0.7% sample prevalence). Three trucks had positive swabs (13.6%) and 4/22 feed samples (18.1%) were positive. Feed and swabs were positive for 2 trucks. More samples were positive from trucks containing meat, bone or fishmeal than those containing vegetable based feed which correlates with a FDA report. Four serotypes were identified and 3/4 are listed in the top 20 serotypes isolated from humans. These data indicate that while the sample prevalence of Salmonella in feed trucks is low (0.7%) the overall contamination rate for feed trucks is much higher (22.7%). Additionally, it may be important to monitor levels of Salmonella in feed transport trucks and devise methods for sanitizing between loads.

Epidemiology of Salmonella spp.: We participated in the NAHMS Cattle on Feed Evaluation to determine the prevalence of Salmonella in feedlot cattle. Within this study, the prevalence of Salmonella species (spp.) in fecal samples was determined. 50 fecal samples were collected from 100 feedlots. Within each feedlot, 25 fresh fecal samples were collected from each of the pen floors of cattle which had been on feed the shortest and longest period of time. Samples were cultured for the presence of Salmonella spp. in both tetrathionate broth (Tet) and GN Hajna broth (GN) and each combined with Rappaports R-10. The total number of samples collected was 4,977; 2,484 and 2,495 from the shortest and longest on feed, respectively. Salmonella spp. were recovered from 38% (38/100) of the feedlots. Salmonella spp. were recovered from 5.5% (273/4,977) of all samples and from 3.5% (88/2,484) and 7.4% (185/2,495) of samples from cattle shortest and longest on feed, respectively. The most common serotype recovered was S. anatum (27.9%), followed by S. montevideo (12.9%), S. muenster (11.8%), S. kentucky (8.2%), and S. newington (4.3%). The most common serogroups identified were E1 (39.6%), C1 (20.7%), and B (10.4%). Shedding of the most common serotypes associated with human illness occurs infrequently (13/273; 4.8%). Of the positive isolates, 77.3% were recovered following culture in both Tet and R-10, while only 49.8% and 36.6% were recovered in Tet alone or GN and R-10, respectively. This study provides information on the status of Salmonella spp. from cattle in feedlots and may serve as baseline information for future studies. We are currently anticipating in the NAHMS Grower/Finisher Swine Survey. approximately 50 fecal samples are being cultured from grower finisher swine on 160 farms to determine the prevalence of Salmonella. Additionally, the finisher ration is also being cultured for Salmonella.

Antimicrobic susceptibility of Salmonella spp.: We are participating with FSIS, APHIS, and FDA to generate baseline antimicrobic susceptibility data from the Salmonella isolates which have been recovered from several NAHMS and FSIS surveys. Specifically, the antimicrobic of interest is the fluoroquinolone as release for use in food animals is imminent. These efforts are being coordinated for use in a national monitoring surveillance program with the FDA, CVM, APHIS, and CDC.

PLAN: Survival of other serotypes of Salmonella in feces will be determined. On-farm surveys and participation in other NAHMS surveys will continue. Antibiograms will be determined for all isolates collected to date in the laboratory. New studies on the tropism of Salmonella heidelberg and Salmonella derby will be initiated.

IMPACT/TECH TRANSFER: This information has furthered our knowledge regarding the carrier state of Salmonella in swine. In addition, nonporcine reservoirs have been identified and their impact on persistence and maintenance of the disease will be determined. This work has been published in scientific journals and presented to commodity groups and other professionals at scientific meetings.

OBJECTIVE C: Define the porcine immune response to acute and chronic Salmonella infection focusing on mechanisms to reduce or eliminate the pathogenic organism

PROGRESS C: Lymphocyte responses following various routes of inoculation with Salmonella spp.: We have shown that high doses of S. choleraesuis administered intranasally cause mitogenic and antigen-specific suppression of lymphocyte proliferation. The significance of in vitro lymphocyte suppression is not clear. However, our data would suggest that this immune dysfunction may play a critical role in the development of the S. choleraesuis carrier state. Development of a stress model to simulate transportation and marketing stress: In collaboration wtth Iowa State University, a series of studies are being conducted to develop a porcine stress model using 2-deoxy-D-glucose (2DG), a metabolic stressor. Results show that 2DG induces a stress response similar to that of rodents (i.e., increased cortisol levels and decreased lymphocyte proliferation). The optimal dose and route for 2DG administration is currently under investigation. Characterization of porcine neutrophil function after S. choleraesuis inoculation: We have developed a modified procedure for the rapid quantitative analysis of phagocytosis by porcine neutrophils using flow cytometry. Preliminary results indicate a transient 2-fold inhibition of phagocytic rate early after infection (day 1 or 2 postexposure). This rate decrease may depend on the age of the pig.

PLAN: We will continue to search for immune dysfunctions associated with salmonellosis in swine. The significance of our most recent observations, suppression of lymphocyte and neutrophil activity, will require further study. Mechanisms by which these phenomena occur will provide important insights as to the best strategy for reduction/elimination of the organism. Once a defined porcine stress model has been established it will be used to determine the effect of stress on the susceptibility of naive and "carrier" swine to salmonellosis.

IMPACT/TECH TRANSFER: Information regarding the potential for immune suppression following high doses of Salmonella will be important to companies involved in vaccine development. Regulation of neutrophil phagocytosis would be a useful method for the control of salmonellosis in swine. Knowledge gained from our stress studies will benefit future studies aimed at reducing or eliminating the potential ill effect(s) of transportation and marketing stress on porcine immunity and subsequent Salmonella spp. shedding at time of slaughter.

OBJECTIVE D: To identify methods to control Salmonella in swine

PROGRESS D: Elimination of Salmonella spp. by use of isolated weaning: We have determined that pigs can be raised free of Salmonella spp. to market weight. Results indicated that pigs kept on the source farm became infected with Salmonella spp., while pigs transported to NADC and raised in isolation remained free of Salmonella spp.

However, data also indicated that without adequate control, pigs raised free of Salmonella will become infected shortly after transfer to finisher sites which have not been adequately cleaned and disinfected. Additionally, we are also developing a Hazard Analysis Critical Control Points plan for use on swine farms. By use of segregated early weaning studies we have also been able to demonstrate a reduction of Salmonella spp. in swine. Currently, we are monitoring swine on several farms from farrow to finish to determine when pigs become infected with Salmonella and if/when they clear Salmonella.

PLANS: New intervention strategies will be designed and tested. On-farm studies have been initiated to determine when Salmonella is first encountered on the farm and factors affecting this appearance will be determined. Based on this information, control measures will be instituted and monitored.

IMPACT/TECH TRANSFER: This information will further our knowledge regarding control and elimination of the carrier in swine. In addition, development of new intervention strategies may also help in the reduction/elimination of the carrier animals. This work has been published in scientific journals and presented to commodity groups and other professionals at scientific meetings.

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CONTROL OF SALMONELLA AND ESCHERICHIA COLI O157:H7 IN LIVESTOCK DURING THE PREHARVEST PERIOD

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GENERAL OBJECTIVE: Reduce Escherichia coli O157:H7 and Salmonella infection in meat animals or meat products using rapid and accurate antemortem and postmortem tests.

OBJECTIVE A: To develop rapid diagnostic tests for E. coli O157:H7.

PROGRESS A: Ten monoclonal antibodies (MAbs) against E. coli O157:H7 antigens, including lipopolysaccharide (O antigen) and flagellar (H antigen) epitopes, have been produced and characterized. These MAbs have been used to develop two different assay formats for E. coli O157:H7 detection: an antigen capture enzyme-linked immunosorbent assay (ELISA) and a membrane antigen rapid test. The antigen capture ELISA uses two different MAbs (of which one is enzyme labeled), has a detection limit of 25,000 bacteria (250,000 bacteria per ml of test material), and can be completed in about 1 hour. The membrane antigen rapid (SMART) test, developed in collaboration with New Horizons Diagnostics Corporation, Colombia, MD, utilizes two MAbs (of which one is colloidal gold labeled), has a detection limit of 500,000 bacteria, and can be performed in about 5 minutes. Based on bacterial growth curves in EC-novobiocin commercial medium, spiked bovine feces and spiked raw hamburger, pre-enrichment of test samples for about 8 hours is necessary for an inoculum of one E. coli O157:H7 bacterium to reach the numerical detection limit in either test format. The antigen capture ELISA format is most suitable for mass screening for E. coli O157:H7 such as in a clinical or diagnostic laboratory setting. The simple and rapid SMART test is probably most suitable for individual clinical or food samples or for working environments such as slaughter plants.

IMPACT/TECH TRANSFER: A prototype test kit (COLI SMART) has been developed in collaboration with New Horizons Diagnostics Corporation. This test has performed well in the laboratory setting and is in the final stages of performance evaluation.

OBJECTIVE B: To develop rapid diagnostic tests for Salmonellae in livestock.

PROGRESS B: To increase likelihood of success, three different approaches to Salmonella detection have been attempted. These are serum anti-Salmonella antibody detection in infected, exposed, or vaccinated animals using recombinant Salmonella proteins; antigen detection via MAb capture immunoassays; and nucleic acid detection with polymerase chain reaction (PCR).

Anti-Salmonella antibody detection: Constructs were made which expressed 50 different recombinant Salmonella proteins, including flagella, outer membrane proteins (OMP-A and OMP-C), and fimbriae. The recombinant proteins were screened by Western immunoblots for reactivity against sera from Salmonella vaccinated or infected animals, including both experimental and persistent naturally infected cattle. Four of 16 recombinant flagella proteins reacted with all Salmonella-challenged cattle sera tested. Recombinant OMP-A (C terminal region) reacted with all Salmonella challenged and vaccinated cattle sera tested, while none of the Salmonella-negative sera were reactive.

Recombinant OMP-C (N and C terminal regions) was also immunogenic and reacted with most infected or vaccinated cattle sera. The recombinant flagella and OMP proteins have potential for use as diagnostic reagents for the serological detection of anti-Salmonella antibodies in infected llivestock.

Antigen detection: Monoclonal antibodies specific for the Salmonella genus; for Salmonella groups B, D, and E; and for Salmonella dublin, S. typhimurium, and S. enteriditis have been produced and characterized. Attempts to format these MAbs as antigen capture ELISAs are under development.

Nucleic acid detection: We have identified a set of OMP-C primers that detect all Salmonella bacteria (52 different serotypes to date) by PCR and does not detect any non-Salmonella bacteria (28 different bacteria tested to date). Nucleic acid sequence analysis showed that the primer sequences are very conserved among the Salmonella serotypes examined so far. The sensitivity of PCR detection in extracted chromosomal DNA was 1 pg. The sensitivity for boiled whole bacteria was 400 cells. Detection in ground beef required 4 to 6 hours of enrichment with an initial inoculum of 100 bacteria.

Field application of Salmonella detection methods to naturally infected cattle: We have located a midwestern commercial dairy cattle herd which is heavily infected with Salmonella. Blood, fecal material, and other clinical samples are being collected over time from these cattle. These samples are currently being tested for the presence of Salmonella antibodies, antigens, and nucleic acids by the methods described above. We hope that this project will permit development of a testing protocol to efficiently identify Salmonella-infected cattle. We are also investigating the immune and infection responses of calves less than 12 weeks of age in this herd--as they appear to respond differently from adult cattle to natural Salmonella exposure and infection.

IMPACT/TECH TRANSFER: We plan to develop a membrane antigen rapid (SMART) test using our anti-Salmonella MAbs via collaboration with New Horizons Diagnostics Corporation (as was done for E. coli O157:H7). In addition, we hope to utilize this same technology and collaboration to produce a single rapid SMART test kit which will detect both E. coli O157:H7 and Salmonella bacteria in food or clinical samples.

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COLIBACILLOSIS IN CATTLE AND SWINE

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OBJECTIVE A: Provide knowledge required to reduce shedding of Escherichia coli O157:H7 in livestock

PROGRESS A: Previously it has been shown that fasting of ruminants can increase fecal shedding of Salmonella and coliforms. This increased shedding is due to changes in rumen pH and volatile fatty acid concentrations. We have previously shown that E. coli O157:H7 grows better in vitro in ruminal contents of fasted animals compared to contents of well-fed animals. As cattle are frequently fasted immediately before slaughter, we examined whether fasting of cattle affected fecal shedding of O157:H7 in vivo. Experimental data demonstrated that cattle that have been fasted for 2 days and then fed are more susceptible to infection with E. coli O157:H7 and shed more O157:H7 than are cattle maintained on full feed. **IMPACT/TECH TRANSFER**: Feeding practices before slaughter are a critical point. Fasted animals should be taken directly to slaughter and not be fed again before slaughter..

OBJECTIVE B: Identify virulence mechanisms of Escherichia coli O157:H7 that permit colonization in gastrointestinal tract of livestock

PROGRESS B: Understanding basic mechanisms of colonization of cattle by O157:H7 will lead to methods that reduce fecal shedding of this human pathogen. It has been shown that O157:H7 colonized the intestinal tract of laboratory animal and causes attaching and effacing lesions in the large intestine . Previously, it was not known where and how O157:H7 colonized the intestinal tract of cattle. We have demonstrated that O157:H7 colonizes the intestinal tract and causes attaching/effacing lesions in colostrum-deprived newborn calves. These attaching/effacing lesions are similar to those seen in experimental infections of rabbits, mice, and gnotobiotic pigs with O157:H7. We are currently determining the effect of colostrum in preventing colonization of calves by O157:H7. If colostrum prevents these lesions and decreases shedding of O157:H7, this may be a method of reducing the level of O157:H7 in young calves.

While cattle have been implicated as an important reservoir for O157:H7 infections in humans, it is not known if other food animals, such as swine, could also be a reservoir. Gnotobiotic pigs are susceptible to infection with O157:H7 but no one has reported if conventional pigs are susceptible are susceptible to such infections. Information on the susceptibility of other animals to O157:H7 is important because non-beef products are also a source of O157:H7 outbreaks in humans. Also, swine and other non-bovine species may be a source of O157:H7 infection in cattle. We have experimentally infected 3-wk-old pigs with O157:H7 to determine if swine are susceptible. These pigs shed low levels of O157:H7 for a 4 weeks, occasionally developed A/E lesions and developed a mild, subclinical disease. **IMPACT/TECH TRANSFER**: The experimental reproduction of attaching/effacing lesions provides insight into the molecular mechanisms involved in colonization of cattle by O157:H7. The experimental data demonstrating that conventional pigs are susceptible to infection with O157:H7 convinced the NAHMS to survey and determine the incidence of O157:H7 in adult pigs in commercial operations.

OBJECTIVE C: Develop vaccines that prevent E. coli infection in domestic animals

PROGRESS C: Induction of an active immune response in older domestic animals should be helpful in reducing colonization by E. coli pathogenic for animals or humans. We have developed 2 live oral vaccines that are effective in preventing postweaning colibacillosis in swine. These vaccines express adhesions, but not toxins,and are therefore safe in addition to being effective. **IMPACT/TECH TRANSFER:** These vaccines will benefit the pork industry by reducing the economic impact associated with disease. A CRADA and BRDC grant have been implemented to allow these vaccines to be transferred to private industry. Our ability to develop safe, effective means of inducing active intestinal immunity in swine for these pig pathogens will prove helpful in our future efforts to develop vaccines for O157:H7 in cattle.

OBJECTIVE D: Characterize strains of E. coli O157:H7

PROGRESS D: We continue to use DNA probes for SLT-I, SLT-II, and eae to characterize isolates from APHIS and FSIS. We are also using animal models to determine if different O157:H7 strains are equally virulent. **IMPACT/TECH TRANSFER:** We have characterized a number of O157:H7 E. coli strains for FSIS, APHIS, and other governmental agencies. Also, we have identified a number of non-O157:H7 coliforms that contain genes associated with human pathogenicity. Serotyping of these non-O157:H7 strains demonstrated that cattle can also carry coliforms with serotype associated with human disease (026, 0111). These strains will be tested in animals to determine if they are pathogenic.

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CONTROL OF CAMPYLOBACTER JEJUNI IN POULTRY

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OBJECTIVE A: To create, produce, and optimize the performance of a vaccine against the flagella of Campylobacter jejuni.

PROGRESS A: We have previously reported the creation and production of a vaccine that was made by DNA engineering. A portion of the flagella gene from Campylobacter jejuni was linked to a protein from another bacteria that stimulates the intestinal immune system. The vaccine was almost 50 percent effective in promoting clearance of C. jejuni from chickens. Efforts to improve the efficacy have been underway but progress was not made until it was discovered that the method we employed for preparing the vaccine caused it to become denatured in such a way as to lose properties essential for immune stimulation. We are currently investigating whether alternative preparation protocols will be less harsh or whether a more crude preparation will be sufficient for vaccination. **IMPACT/TECH TRANSFER:** This vaccine can easily be applied and has potential as a method of controlling C. jejuni. A patent has been applied for this vaccine.

OBJECTIVE B: Identify components of C. jejuni that the organism requires for inhabiting the intestines of chickens and may serve as vaccine candidates.

PROGRESS B: We previously reported finding proteins produced by C. jejuni in which the form of the protein produced correlated with the ability of the organism to inhabit the intestines of chickens. We have cloned one of these proteins, which we have designated ompH1. Sequencing of the protein has been completed and the protein is highly similar to a binding protein that has been correlated to infectivity of other bacteria, the glutamate binding protein. Also, there was no difference in the protein sequences between the strains that could and could not inhabit the chicken intestines. This indicates a difference in protein modification systems. **IMPACT/TECH TRANSFER:** These findings have direct applied impact in that components of the bacteria with potential for vaccine use have been identified. The findings also add to our scientific knowledge of the biology of how intestinal pathogens avoid the immune system.

OBJECTIVE C: To determine the degree of variability of the flagella genes of C. jejuni and evaluate for effect on vaccine performance and for potential on tracking strains of C. jejuni in epidemiological studies.

PROGRESS C: The sequence of the primary flagella gene for eight strains of C. jejuni was determined and analyses were made with these data plus data from four more strains found in publications. The analyses revealed two major groups of strains. Most variation was near but not in areas of the protein that are most immunogenic. A vaccine with two types may be sufficient to cover the species. In addition, it was found that sequencing of short pieces of DNA that could be done in one or two reactions yields enough information to discriminate strains at a level equivalent or better than serotyping. The advantage over serotyping is that the reagents that are used for sequencing are universally available. **IMPACT/TECH TRANSFER:** These findings will help to design

modifications to the vaccine mentioned before so that all strains of Campylobacters will be inhibited. Analyses of this data will also add to our fundamental knowledge of why bacteria carry two copies of a given gene.

OBJECTIVE D: Track the sources of Campylobacters for broiler colonization. Answer why Campylobacters only colonize poultry after two or three weeks into production. Indicate the role of colonizing but not (yet) cultivable (CNC) Campylobacter spp, in transmission to poultry flocks.

PROGRESS D: We have previously reported developing and applying restriction fragment length polymorphism (RFLP) analysis of Campylobacters for potential tracking of isolates. We objectively discriminate between isolates and determine sources in their transmission. Using RFLP, we determined that Campylobacter spp. from several environmental sources colonize broiler chickens during production. Samples of insects, warm blooded animals (both rodents and livestock animals), and farmers' boots carried flaA types that were indistinguishable from those isolates found colonized by broilers. We continue our cooperation with DuPont, Inc. to assess potential applications of ribotyping analysis. We are continuing our studies to define whether CNC Campylobacter spp. can be transmitted through spent litter and/or other fomites. **IMPACT/TECH TRANSFER:** This work will lead the poultry industry toward identifying the critical control points and potential areas for intervening in the presence of Campylobacters.

OBJECTIVE E: Determine whether all Campylobacters associated with poultry are potential human pathogens.

PROGRESS E: Cooperatively, with the Royal Melbourne Institute of Technology, we observed that about 10% of the Campylobacter spp. from our poultry isolates exhibit DNA sequences which are consistent with those DNA profiles associated with human disease. The DNA profile was obtained by using DNA probe pMO2005 and was digested by the restriction digest enzyme ClaI. The most common Campylobacter (78%) profile isolated from poultry sources yielded a two-band profile (14.5 kb and 4.0 kb) while a single-band profile (18.5 kb) was typical of the majority (71%) of human strains. There is a need to assess whether this relation is consistent among poultry and human isolates and whether poultry isolates are or are not of equal pathological consequence for humans. **IMPACT/TECH TRANSFER:** This work will determine whether all Campylobacters are of equal public health concern and could lead toward competitive exclusion of the human pathogenic strains.

OBJECTIVE F: Determine whether defined antagonistic micro flora can be used to reduce colonization by human infecting Campylobacter spp. Determine mechanisms involved. Optimize treatment(s) and monitoring capabilities.

PROGRESS F: We used Saccharomyces cerevisiae var. boulardii (SB) for controlling colonization of broiler chicks with Campylobacter spp. The frequency of chick colonization was reduced from 90% in positive control chicks to 40% in the SB treated birds. Analysis of colonized chicks revealed a 97% decrease in Campylobacters in the SB treated chicks. We are attempting to determine whether the mannose molecules expressed on the surface of SB is able to attract and agglutinate Campylobacter spp. within the chicken intestinal tract. Such agglutination could explain the colonization reduction by intestinal flushing through peristaltic movement. As we understand the mechanism involved, we hope to enhance such reduction in the chicken. We are currently assessing alternative means for administering yeast to optimize reduction of Campylobacter spp. in an economical manner. **IMPACT/TECH TRANSFER:**This work provides a defined, safe, and low

technology method for the reduction of this most important poultry borne human pathogen.

OBJECTIVE G: Determine the practicality of imposing biosecurity as a means to control Campylobacter spp. Determine which critical CONCERN points have greatest influence on levels of Campylobacter spp. associated with poultry carcasses.

PROGRESS G: We have previously reported that incorporating 5 p.m. chlorine in commercial broiler drinker waters does not diminish the incidence of Campylobacters in flocks relative to the incidence in paired houses not receiving treatment. We also report that transport of broilers from production to processing facilities multiplies levels of the organism found on carcasses by about 100 fold. These two observations support the contention that broilers are likely to be exposed to Campylobacter spp. and, numbers multiplied during production and transport. **IMPACT/TECH TRANSFER:** By imposing intervention strategies designed to reduce levels of bird colonization we hope to decrease the numbers on the fully processed carcasses and, diminish human exposure. Thus, biosecurity as a means to eliminate the organism should be discounted and, rather, emphasis should be placed on diminishing the levels of the organism on the poultry product.

OBJECTIVE H: Create improved methodology for detection and enumeration of Campylobacter spp.

PROGRESS H: We are currently determining whether optimum media compositions can be described which allow for selection of Campylobacter spp. under normal atmospheric conditions. We are exploring the applicability of the Vitek-bioMerieux Vidas system for the automated detection of Campylobacter spp. in one day less analysis time. **IMPACT/TECH TRANSFER:** These studies should allow for simplified culture and detection of Campylobacters in food microbiology laboratories both in the private and public sectors.

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PREHARVEST DETECTION IN LIVESTOCK OF POTENTIAL HUMAN FOODBORNE PATHOGENS: LISTERIA MONOCYTOGENES, CAMPYLOBACTER, ARCOBACTER, AND YERSINIA

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OBJECTIVE A: Develop rapid and sensitive detection techniques.

PROGRESS A: Multiplex PCR assays have been developed to (a) differentiate Listeria monocytogenes from other Listeria species, (b) distinguish C. jejuni from C. coli, and (c) rapidly differentiate Arcobacter from other fastidious, Campylobacter-like organisms. Conventional methods to detect and to identify Campylobacter involve lengthy incubations and specialized gas mixtures. Additionally, the hippurate hydrolysis test to differentiate between C. coli and C. jejuni is unreliable. A multiplex polymerase chain reaction (mPCR) assay was developed for the detection of Campylobacter from swine rectal swabs. The assay can detect C. coli/jejuni and differentiate between the two species. Two sets of primers are used in this assay. The first set amplifies a portion of the flagellin genes of both C. coli and C. jejuni. The second set of primers amplifies a DNA target found only in C. jejuni. Based on the band pattern resulting from electrophoresis of the mPCR product, one can determine the presence of either C. coli or C. jejuni and also differentiate between these two organisms. Arcobacter spp. are fastidious spiral organisms which are closely related to campylobacters and helicobacters. PCR primers specific for the 16S RRNA are being used as a rapid screen in hog prevalence surveys to identify Arcobacter directly from primary enrichment broths. Only PCR-positive samples are processed further for bacterial isolation, eliminating the need to process each primary culture. The resultant savings in material and labor are considerable.

An mPCR assay was developed for Listeria, to determine the presence of Listeria and, simultaneously, to identify L. monocytogenes. Two sets of primers are used. One amplifies a segment in all Listeria tested; the other targets a portion of DNA found only in L. monocytogenes. Based on the electrophoretic pattern of the resultant mPCR product, one can determine the presence of Listeria spp. and also differentiate L. monocytogenes. PCR primers specific for L. monocytogenes unequivocally identify colonies of L. monocytogenes without the need for extensive DNA purification. Correct identification is especially critical in light of current "zero tolerance" standards for this pathogen.

IMPACT/TECH TRANSFER: Multiplex PCR is a rapid and sensitive method to screen animals for the presence of food-borne pathogens. The transfer to FSIS of multiplex PCR technology for the detection from enrichment broth of C. jejuni/coli is currently under negotiation. The transfer to FSIS of multiplex PCR technology for L. monocytogenes is planned.

OBJECTIVE B: Define the epidemiological roles of livestock and poultry as carriers of potential food-borne pathogens.

PROGRESS B: Previously, we reported recovery of significantly more Arcobacter from aborted porcine fetuses (n=400 samples, 47% positive) than from normal fetuses (n=200 samples, 22% positive). In another study, we cultured Arcobacter from 84% of hog farms

surveyed. We have now surveyed 5 ground pork plants for the presence of Arcobacter. Recoveries ranged from 0-90% positive ground pork samples. Plant hygiene and/or swine rearing practices may explain differences in the rate of contaminated pork products. Previous researchers have reported Campylobacter jejuni and Helicobacter pylori readily colonize neonatal piglets. We have used the neonatal piglet model to determine the pathogenicity of Arcobacter. A. butzleri is the Arcobacter species most frequently recovered from clinically ill patients. Piglets infected with either A. butzleri or A. cryaerophilus 1B shed the organisms in their feces until necropsy. However, only A. butzleri could be recovered from tissues. In contrast, animals experimentally infected with A. skirrowii or A. cryaerophilus 1A shed the organisms briefly. This is the first report of colonization of experimentally infected piglets by Arcobacter spp. and emphasizes the pathogenicity of A. butzleri.

IMPACT/TECH TRANSFER: The identification of Arcobacter spp. in swine and the susceptibility of piglets to this agent signals the emergence of a potential food-borne concern.

OBJECTIVE C: Evaluate management practices to reduce or eliminate zoonotic food-borne pathogens.

PROGRESS C: We are participating in a study to evaluate segregated early weaning (SEW) as a management technique to reduce zoonotic food-borne pathogens in pigs. We wished to determine if the prevalence (Campylobacter jejuni, Campylobacter coli, Arcobacter spp. and Listeria monocytogenes) was lower in pigs raised by SEW than with on-farm cohorts. Piglets were sampled as they entered the SEW nursery (approx. 3 wk of age), as they exited approximately 4-5 wk later, and at market weight. Rectal swabs were cultured for Campylobacter, Arcobacter, and Listeria monocytogenes. Preliminary data, based on the percentage of positive farms, indicate that piglets are positive for Campylobacter despite routine antibiotic therapy administered as they entered the nursery and a diet of medicated feed. No differences were seen in the percentage of farms positive for Campylobacter in the SEW (38%) vs. control (41%) herds. However, the prevalence of Arcobacter, as determined by the percentage of positive farms, was higher for the SEW premises (84%) than for the on-farm controls (24%). Although Listeria innocua was recovered, L. monocytogenes was not found in either the SEW or control herds. In FY 96, scientists will complete a second evaluation of the impact of SEW on reducing the level of C. jejuni/coli and Arcobacter spp. in hogs.

IMPACT/TECH TRANSFER: The segregated early weaning (SEW) study indicates that a management system which reduces the prevalence of one pathogen may not guarantee a decline in all potential food-borne agents The 1995 Swine USDA-APHIS-National Animal Health Monitoring Survey involves the 16 major pork-producing states and nearly 8,000 animals. In FY 96, we will continue to survey for Campylobacter spp. and Yersinia as part of that study. The prevalence of these two pathogens will be correlated with herd size and management system. Campylobacter jejuni is a cause of sheep abortion as well as a human food-borne pathogen. We will anticipate in an APHIS study to determine the prevalence of this pathogen in sheep (n=4,000) raised on pastures vs. feedlots. Because of the association of sheep with food-borne outbreaks of Listeria monocytogenes, we will also determine the prevalence of this pathogen in sheep.

IMPACT/TECH TRANSFER Prevalence surveys conducted in association with APHIS will provide needed data on prevalence of food-borne pathogens in U.S. herds.

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DIAGNOSIS AND EPIDEMIOLOGY OF BOVINE TUBERCULOSIS

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OBJECTIVE A: Develop improved tests for diagnosis of bovine tuberculosis.

PROGRESS A: Tuberculin skin tests are the primary tests used for preharvest diagnosis of bovine tuberculosis. Tuberculins used for skin testing in Canada, Mexico, and the United States are prepared by different methods and from different strains of Mycobacterium bovis. We compared tuberculins from each country to determine if there is a need to standardize tuberculins used in North America. Cattle from a herd with bovine tuberculosis reacted similarly to all three tuberculins with a small amount of variation observed in responses. Cattle were test positive most often when the tuberculin from Mexico was used. However, there does not appear to be a need to standardize tuberculins used in North America. These findings are important with recent passage of the North American Free Trade Agreement and increased movement of cattle between countries.

OBJECTIVE B: Development of an animal model of human infection with Mycobacterium bovis.

PROGRESS B: Swine have been used as an animal model to study a variety of human diseases. We conducted research to determine if swine can be used as an animal model of human tuberculosis. Through a series of experiments, we determined that swine are susceptible to infection with M. bovis when challenged by the intratracheal, intratonsilar, intravenous, intragastric, and oral routes of inoculation. Doses used for inoculation ranged from 10^8 to 10^2 colony forming units of M. bovis. Lesions characteristic of tuberculosis were visible as early as 2 weeks when high doses were used and at 30 days when lower doses were used. We are continuing to conduct experiments to further characterize tuberculosis in the animal model. **IMPACT/TECH TRANSFER:** We will use swine in research to study the risk of M. bovis infection caused by consumption of meat contaminated with the organism.

PUBLICATIONS:

Goff, B.S., Angus, D., Bojorquez Narvaez. L., Bolin, C.A., Gurria Trevino, F., Jarnagin, J., Martin, B., Martinez Luken, G., Meyer , B., Payeur , J., Pena Flores, G., Rohonczy, E., Saari , D., Whipple, D.L. 1995. Comparison of North American PPDs in vivo: Caudal Fold Testing. Proceedings of Intl. Symp. on Bovine Tuberculosis in Animals and Human Beings. May 1995, College Park, MD. (In Press).

Bolin, C.A., Whipple, D.L., Gustafson, K., Risdahl, J., Stabel, J.R., Molitor, T. 1995. Development of a model of Mycobacterium bovis infection in swine. Proceedings of Intl. Symp. on Bovine Tuberculosis in Animals and Human Beings. May 1995, College Park, MD. (In Press). Diagnosis and Immunology of Paratuberculosis (Johne's) of Cattle.

OSTEOMYELITIS-SYNOVITIS IN TURKEYS

ARS Contact Persons:
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CRIS Number: 6226-32000-002 -
Termination Date: June 1996
FSIS Number: I-92-2

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OBJECTIVE A: To determine the etiology of turkey green-liver/osteomyelitis complex (TOC) and formulate management practices that will lead to the elimination of the problem.

PROGRESS A: USDA-ARS research on turkey osteomyelitis complex (TOC) is focused on determining why a small percentage of processed turkeys are infected with a variety of opportunistic bacteria, affecting not only bone and synovial tissue, but also causing deep muscle abscesses. We previously reported the results of a field study which determined that TOC apppears to be a chronic infection, associated with air-sacculitis, pericarditis, and that many different bacterial species can be isolated from the lesions. We have also found that cell-wall deficient (CWD) bacteria are often isolated from TOC lesions, including those which appear sterile using traditional culture methods. This observation explains the previously reported lack of bacterial isolation from many TOC lesions, since CWD bacteria require stringent growing conditions. It also led to the current hypothesis that TOC is the result of host failure to completely destroy opportunistic bacterial pathogens and that differences in the immune response of a certain population of turkeys may be involved. This study also demonstrated that the incidence of tibial dyschondroplasia (TD) follows the same time course as does TOC incidence, and that the severity of TD in 14-15 week old turkeys is correlated with increased body weight. Hemorrhagic lesions within the TD cartilage were described which may lead to focal infection.

IMPACT/TECH TRANSFER: This study determined that piperazine, the only antihelmentic approved for use in turkeys, was ineffective in preventing infestation with Ascaridia dissimilis. This information led to official meetings with the FDA to encourage the licensure of other anthelmentics for use in turkeys.

OBJECTIVE B: To determine whether inate or acquired immune deficiency may account for the susceptibility of a small population of turkeys to infection with opportunistic bacterial pathogens.

PROGRESS B: We have established that turkeys with TOC lesions have significantly lower T-lymphocyte responses in both in vivo and in vitro tests, and have higher complement activity than do healthy turkeys. These results parallel the immune dysfunctions reported for human chronic osteomyelitis. This investigation has led to further study of the inate immune responses of turkey poults in hopes of distinguishing populations with relatively low T-cell functions, and ultimately discerning the specific functional defect responsible for the lowered response. We have also completed a study comparing the T-lymphocyte functions of processing-age turkeys genetically selected for increased body weight or for increased egg production with their random-bred control parent lines and with two commercial turkey strains. Significantly lower toe-web hypersensitivity in the line selected for increased body weight and in the commercial line with a reportedly higher susceptibility to TOC, suggests that turkey breeders may be inadvertantly selecting for lower T-cell response when selecting for faster growth rate.

IMPACT/TECH TRANSFER: If an inate immune dysfunction is shown to be characteristic of those birds that subsequently develop TOC lesions, geneticists may be able to eliminate those birds from the breeder flocks. Nutritional intervention may also help to compensate for immune impairment without decreasing productivity.

OBJECTIVE C: To determine the cause of green livers in turkeys which do not have TOC lesions.

PROGRESS C: The FSIS screening program for TOC relies on the presence of a green liver as an indicator of infection. However, only about 50% of the turkeys with green livers have TOC lesions. We examined the effects of feed and water withdrawal on the occurence of green livers in processing-age turkeys. While it is commonly assumed that the withdrawal of feed before birds are processed causes green liver due to bile stasis, this study saw no association between feed withdrawal up to 54 hours prior to processing and green liver discoloration.

IMPACT/TECH TRANSFER: The occurence of green livers in turkeys without TOC lesions is a significant ecomonic problem to the turkey industry. This research was successful in breaking down an intellectual barrier and thus redirecting efforts in determining the causes of green liver in turkeys without obvious infection.

PUBLICATIONS:

Bayyari, G.R., W.E. Huff, R.A. Norton, J.K. Skeeles, J.N. Beasley, N.C. Rath, and J.M. Balog. 1994. A longitudinal study of green liver-osteomyelitis complex in commercial turkeys. Avian Dis. 38:744-754.

Bayyari, G.R. 1995. USDA-ARS research on turkey green-liver osteomyelitis complex (TOC). Proceedings of the Arkansas Nutrition Conference. .

Norton, R.A., G.R. Bayyari, J.K. Skeeles, W.E. Huff, and J.N. Beasley. 1994.. A survey of two commercial turkey farms experiencing high levels of liver foci. Avian Dis. 38:887-894.

Bayyari, G.R., W.E. Huff, N.C. Rath, J.M. Balog, L.A. Newberry, and J.K. Skeeles. 1995. Immunological characterization of turkeys with green-liver osteomyelitis complex and normal turkeys. Southern Poultry Science Society Annual Meeting. Atlanta, GA.. (Abstract)

Huff. W.E., G.R. Bayyari, J.M. Balog, and N.C. Rath. 1995. Failure of feed and water withdrawal to cause mimicry of the green livers associated with turkey osteomyelitis complex. Southern Poultry Science Society Annual Meeting. Atlanta, GA. (Abstract)

STRATEGIES TO CONTROL SWINE PARASITES AFFECTING FOOD SAFETY

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CRIS Number: 1265-32000-049
Termination Date: Sept. 2000
FSIS Number: N/A

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OBJECTIVE A: Reduce transmission of foodborne pathogens of swine by defining cytokine- regulated immune mechanisms that protect pigs against parasites that threaten food safety.

PROGRESS A: 1) Assessed protective immune responses of swine against Toxoplasma gondii in an effort to decrease incidence of swine toxoplasmosis and thus prevent human disease. With National Pork Producer Council funding, developed reagents to quantitate swine cytokine production during parasitic infections. 2) Analyzed synergistic interactions between the whipworm, Trichuris suis parasite and enteric bacteria (Campylobacter spp.) in producing severe pathology in the colon of growing pigs. Determined that bacterial overgrowth in growing pigs with Trichuris suis infections can be controlled by antibiotic treatment or vaccination against whipworm.

IMPACT/TECH TRANSFER A: 1) Used this basic knowledge of protective immune responses against Toxoplasma gondii to aid in design and evaluation of candidate vaccines for this infection. 2) Verified that Trichuris suis infections can cause serious colonic disease in pigs and, more importantly, can set the stage for colonization of bacterial infections that affect food safety.

OBJECTIVE B: Reduce transmission of foodborne pathogens of swine by identifying DNA sequences for diagnosis and for expression of specificand shared antigens for a broad spectrum anti-worm vaccine.

PROGRESS B: 1) Developed DNA probes that differentiate Trichinella species that infect domestic swine from those that are freeze resistant. Determined that two distinct species of Trichinella can infect the same host simultaneously. Research is now directed toward determining whether hybridization can occur between the freeze resistant and non-freeze resitant genotypes. 2) A proteolytic enzyme which causes the formation of lesions in the swine colon, leading to secondary bacterial infection, was cloned from the whipworm, Trichuris suis parasites.

IMPACT/TECH TRANSFER B: 1) Successfully identified species of Trichinella causing disease in several wild animal infections. 2) The cloned Trichuris suis protein will be used in inhibition and immunization studies to test its efficacy in a multicomponent vaccine against swine parasites. With National Pork Producer Council funding, feed efficiency will be analyzed during these trials.

PUBLICATIONS:

Hill, D.E., Fetterer, R.H., Romanowski, R.D., & Urban, J.F. 1994. The effect of immunization of pigs with Ascaris suum cuticle components on the development of resistance to parenteral migration during a challenge infection. Vet. Immunol. & Immunopath. 42, 161-169.

Mansfield, L.J. & Urban, J.F. 1995. The pathogenesis of necrotic proliferative colitis in swine is linked to whipworm induced suppression of mucosal immunity to resident bacteria. Vet. Immunol. & Immunopath.(In Press)
Zarlenga, D.S. 1994. The application of repetetive DNA probes in assessing the potential for hybridization between Trichinella nativa and Trichinella spiralis. Proc. Intnl. Conf, Trichinellosis. 8:117-122.

Baker, D., Bryant, J.D., Urban, J.F., & Lunney, J.K. 1994. Swine immunity to selected parasites. Vet. Immunol. & Immunopathol. 43:127-134.

PREVENTION & THERAPY FOR PROTOZOAN PARASITES AFFECTING FOOD ANIMALS, FOOD SAFETY, PUBLIC HEALTH

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CRIS Number: 1265-32000-050
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OBJECTIVE: Prevent illness in food animals, control food contamination and protect public health from protozoan parasites. Cryptosporidium parvum affecting all mammals will be a target of immunity based strategies.

PROGRESS: (1) The first direct genetic immunization of livestock using parasite genetic material from Cryptosporidium resulted in a detectable immune response. (2) The first use of the cytokine IL-12 against Cryptosporidium was highly effective in preventing infection in test animals. (3) Time versus temperature determinations revealed that the infectious stage of Cryptosporidium can be inactivated after 1 minute at 72.4 C. (4) Exposure of Cryptosporidium to concentrated laundry bleach did not inactive the parasite.

IMPACT/TECH TRANSFER: (1) and (2) New methods for immunization and disease prevention were discovered. Collaborators in industry have expressed interest in pursuing these methods for commercialization. (3) The exact time and temperature for disinfection of water greatly reduces the cost and increases the potential for disinfection compared with previous requirements to boil water for 5 to 20 minutes. (4) The determination that concentrated bleach was an ineffective disinfectant for homes, laboratories, medical and veterinary facilities provided important information to the medical community and prompted the Clorox Company to initiate a collaborative project to test new disinfectants.

PUBLICATIONS:

Fayer, R. 1994. Foodborne and waterborne zoonotic protozoa. Chapter 12. In: Foodborne Disease Handbook. Vol. 2. Eds. Hui, Y.H., Gorham, J. R., Murrell, K. D. and Cliver, D. O. Marcel Dekker Publ. Inc. New York. 331-361.

Fayer, R. 1994. Effect of high temperature on infectivity of Cryptosporidium parvum oocysts in water. Applied and Environmental Microbiology 60:2732-2735.

Zu, S.-X, Li, J. F., Barrett, L. J., Fayer, R., et al. 1994. Seroepidemiologic study of Cryptosporidium infection in children from rural communities of Anhui, China and Fortaleza, Brazil. American Journal of Tropical Medicine and Hygiene 51:1-10.

Fayer, R. and Ellis, W. Qinghaosu (artemisinin) and derivatives fail to protect neonatal BALB/c mice against Cryptosporidium parvum (Cp) infection. Journal of Eukaryotic Microbiology 41:41S. 1994.

Fayer, R. 1994. Development of a precocious strain of Cryptosporidium parvum in neonatal calves. Journal of Eukaryotic Microbiology 41:40S.

Fayer, R. 1995. Effect of sodium hypochlorite exposure on infectivity of Cryptosporidium parvum oocysts for neonatal BALB/c mice. App. & Environ. Microbiology 61:844-846.

IDENTIFICATION AND MAPPING OF GENES INVOLVED IN PARASITIC DISEASE RESISTANCE/SUSCEPTIBILITY

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OBJECTIVE: Identify and breed livestock and poultry that are genetically resistant to parasite infections.

PROGRESS: 1) Tested genetically defined minipigs, specifically those with swine leukocyte antigens of a haplotype, for factors that enable Trichinella spiralis infected pigs to reactagainst encysted stage of this parasite. 2) Began studies to determine whether Toxoplasma gondii resistant pigs can be identified and to define the genes and immune factors that encode such parasite resistance.

IMPACT/TECH TRANSFER: These studies should help breeders to reduce costs of drug and vaccine treatments by selecting for parasite resistant stock.

PUBLICATIONS:

Lunney, Joan K. 1994. Genetic Markers for Disease Resistance. Proceedings NCR150 Meeting, St.Louis. pp.35-42.

Lunney, J.K. 1994. The Swine Leukocyte Antigen (SLA) Complex. Vet. Immunol. Immunopathol. 43:19-28.

Lunney, J.K., and Grimm, D.R. 1994. The Major Histocompatibility Complex: Current state of knowledge and its use in and impact on livestock improvement. Proc. 5th World Congr. Genet. Appl. Livest. Prod., Guelph, Canada. 20: 230-237.

EPIDEMIOLOGY AND CONTROL OF TRICHINAE IN THE NORTHEAST

ARS Contact Person:
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CRIS Number 1265-32000-045
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OBJECTIVE A: To validate the use of the ELISA test as a tool for identifying trichinae-infested premises.

PROGRESS A: Several experiments have been performed to verify the performance of a commercial ELISA which is being evaluated in the Ohio state veterinarians laboratory as part of the project. The LMD Trichinella Serology Microwell kit performed as well as the ARS research ELISA in detecting infected pigs and in reducing background. A comparison of these tests is submitted for publication (Gamble and Patrascu, 1995). Testing of 20,000 sera using the commercial test kit is ongoing in the Ohio lab and verification is being performed in the ARS, Beltsville lab. The results of this portion of the study will be validation of the commercial test kit for trichinellosis in pigs which can then be used in the industry effort to monitor the production of trichinae-free pigs. In addition to the Ohio effort, testing of swine farms in New Jersey and New England using the ELISA test is ongoing, with approximately 1/2 of the projected 180 farm tested. Follow-up on positive samples includes confirmation of results by digestion testing. To date, we have found one confirmed positive premise and two other suspect locations which are being evaluated in greater depth. Additional slaughter samples are being tested with occasional positive results. **IMPACT/TECH TRANSFER**: Completion of this portion of the study will provide an updated prevalence of swine trichinellosis for this area of the country and will verify the usefulness of the ELISA test for evaluating the presence of trichinellosis on-farm.

OBJECTIVE B: To associate risk factors with the presence of trichinae in pigs (for on-farm control programs).

PROGRESS B: Data has been acquired from all premises tested in New Jersey and New England, where approximately 1/2 of the total work has been completed. Farm data has been entered into EpiInfo and will be used to evaluate the risk factors for trichinellosis at the conclusion of the testing period. The results of this work will provide risk factor profiles which can be used to establish conditions under which pigs can be raised trichinae-free. However, because only a limited number of positive premises have been identified to date, progress is slow.

OBJECTIVE C: To validate the efficacy of the ELISA test for swine trichinellosis in large scale field trials.

PROGRESS C: This is a cooperative project between ARS. LMD Laboratories, Inc. (a private company), and the government of Romania. A high rate of human trichinellosis is found in Romania and this is an ideal location for large scale field testing of the ELISA in pigs. ARS scientists will work with the cooperators to provide a highly sensitive version of the ELISA test for use in Romania. The ARS cooperator visited the Romanian study site and developed plans for the next two years. Romanian veterinary inspectors will select two study areas with high and low prevalence of trichinellosis. On-farm testing will be performed using the LMD Trichinella Microwell Serology ELISA. All tested pigs, positive and negative, will be followed to slaughter and samples collected for digestion

testing. The results of this study will be a large data set demonstrating the efficacy of serology testing for trichinellosis in swine which can be used in determining the applicability of serology testing in the U.S. and Europe.

PUBLICATIONS:

Gamble, H.R. and Patrascu, I.V. 1995. Use of blood and tissue fluids in a serum-based ELISA for swine trichinellosis. Journal of Food Protection (submitted)

CHECK SAMPLE PROGRAM FOR TRICHINAE INSPECTION OF HORSEMEAT

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CRIS Number: 1265-32000-045
Termination Date: March, 1999
FSIS Number: N/A

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OBJECTIVE A: To provide training and quality control to a program for the inspection of horsemeat (and pork) for export to the EU.

PROGRESS A: The ARS administers a training program for the certification of trichinae analysts in horse, and more recently, swine slaughter facilities. The program is accepted by the European Union and allows U.S. horsemeat and pork to be certified trichinae free for export. Two or more training sessions are held each year for personnel from participating plants to become certified. On a quarterly basis, check samples are prepared and distributed to all certified trichinae analysts for testing. Accurate analysis of these check samples allows for continued certification of these inspectors.

OBJECTIVE B: To provide data supporting the efficacy of current and proposed methods for the inspection of horses and pigs for trichinellosis.

PROGRESS B: A research project to determine the effectiveness of digestion and serology methods for detection of trichinellosis in horses was undertaken jointly by ARS scientists and scientists from Agriculture Canada. Horses were given various doses of Trichinella spiralis then slaughtered and tissues and serum tested using methods approved by the EU and USDA. These studies demonstrated that the tongue and masseters were the best muscles to sample using pooled digestion methods and that a 5 or 10 gram sample size was required to assure that there was no public health risk associated with digestion negative animals. One gram samples, as mandated for inspection of pigs in the EU was not acceptable. Serology testing by ELISA was as effective as the digestion methods for detecting horses with trichinellosis. The results of this study will be used by the USDA and the EU to clearly define the requirements for inspection of horses for trichinellosis. A similar study, with similar results was conducted in pigs.

PUBLICATIONS:

Gamble, H.R. 1995. Detection of trichinellosis in pigs by digestion and enzyme immunoassay. Journal of Food Protection (In Press).

Gamble, H.R., Gajadhar, A.A. and Solomon, M.B. 1995. Detection methods for trichinellosis in horses. Journal of Food Protection (In Press).

PORCINE TOXOPLASMOSIS NATIONAL PREVALENCE

ARS Contact Person:
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CRIS Number: 1265-32000-045
Termination Date: March, 1999
FSIS Number: I-89-94

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OBJECTIVE: Determine the prevalence of toxoplasmosis in swine and develop control procedures.

PROGRESS: In a serologic survey, 23.9% of 11,842 commercial pigs slaughtered in 1983-84 throughout the United States had antibodies to Toxoplasma gondii by the use of a modified agglutination test (MAT). Follow up field studies were initiated in 1992 on swine farms in Illinois to determine risk factors associated with toxoplasmosis in hogs. These results are summarized here.

Field studies were conducted on 47 swine farms in Illinois to identify sources and reservoirs of Toxoplasma gondii infection. Blood samples were obtained from swine and from trapped wildlife. Serum antibodies to T. gondii were determined using the MAT, incorporating mercaptoethanol. Antibodies to T. gondii (titer > 25) were found in 97 of 4,252 (2.3%) finishing pigs, 395 of 2,617 (15.1%) sows, 267 of 391 (68.3%) cats, 126 of 188 (67.0%) raccoons, 7 of 18 (38.9%) skunks, 29 of 128 opossums (22.7%), 6 of 95 (6.3%) rats, 3 of 61 (4.9%) white-footed mice (Peromyscus sp.), and 26 of 1,243 (2.1%) house mice (Mus musculus). Brains and hearts of rodents trapped on the farm were bioassayed in mice for the presence of T. gondii. Toxoplasma gondii was recovered from tissues of 7 of 1,502 (0.5%) house mice, 2 of 67 (3.0%) white-footed mice and 1 of 107 (0.9%) rats. Feces of 274 cats trapped on the farms and samples of feed, water, and soil were bioassayed in mice for the presence of T. gondii oocysts. Toxoplasma gondii was isolated from 2 of 491 (0.4%) feed samples, 1 of 79 (1.3%) soil samples, and 5 of 274 (1.8%) samples of cat feces. All mammalian species examined were reservoirs of T. gondii infection. All farms had evidence of T. gondii infection either by detection of antibodies in swine or other mammalian species, or by detection of oocysts, or by recovery from rodents by bioassay. The possibility of transmission of T. gondii to swine via consumption of rodents, feed, and soil was confirmed.

Two epidemiologic studies of risk factors for transmission of Toxoplasma gondii to swine were conducted for farms in Illinois. The first study was a sectional survey of swine farms from the state of Illinois pseudorabies testing program, in which farm owners or managers were interviewed by telephone regarding presence of risk factors for transmission of T. gondii on the farm. There were 123 farms surveyed which provided blood samples for at least 30 sows. The mean sow seroprevalence was 19.5% (median - 10.0%). Multiple regression analysis of the association of sow seroprevalence with outdoor housing of sows, cat access to sow areas, number of sows, open feed storage and water delivery, delayed removal of carcasses, and presence of rodents on the farm indicated that higher sow seroprevalence was associated with cat access to sows ($P = 0.009$) and fewer sows in the herd ($P = 0.05$). The second study was a field investigation of 47 swine farms (37 from the cross-sectional study). Data collection included obtaining blood samples from swine, cats and rodents, and fecal samples from cats, heart and brain tissue from rodents, and feed, water, and soil samples for T. gondii examination. The risk of T. gondii transmission from cats and rodents to sows and finishing pigs was evaluated, taking into account housing conditions and herd size. Multiple regression analysis indicated that T. gondii seroprevalence in finishing pigs increased with more seropositive juvenile cats on

the farm ($P < 0.0001$), and higher seroprevalence in house mice ($P = 0.00235$). For sows, the only risk factor associated with increased T. gondii seroprevalence was a higher number of seropositive juvenile cats on the farm ($P = 0.0008$). Housing swine outdoors was not associated with a higher T. gondii seroprevalence. **IMPACT/TECH TRANSFER:** These results identify T. gondii infection in cats (particularly juveniles) and house mice as indicators of increased risk of transmission to swine.

PUBLICATIONS

Dubey, J.P., Thulliez, P., and Powell, E.C. 1995. Toxoplasma gondii in Iowa sows: Comparison of antibody titers to isolation of T. gondii by bioassays in mice and cats. J. Parasitol. 81: 48-53.

Dubey, J.P., Thulliez, P., Weigel, R.M., Andrews, C.D., Lind, P. and Powell, E.C. 1995. Sensitivity and specificity of various serologic tests for the detection of Toxoplasma gondii infection in naturally infected sows. Am. J. Vet. Res. 56:1030-1036.

Weigel, R.M., Dubey, J.P., Siegel, A.M., Kitron, U.D., Mannelli, A.M., Michell, M.A., Mateus-Pinilla, N.E., Thulliez, P., Shen, S.K., Kwok, O.C.H., and Todd, K.S. 1995. Risk factors for transmission of Toxoplasma gondii on swine farms in Illinois. J. Parasit.

Dubey, J.P., Weigel, R.M., Siegel, A.M., Thulliez, P. Kitron, U.D., Mitchell, M.A., Mannelli, A.M., Mateus-Pinilla, N.E., Shen, S.K., Kwok, O.C.H., and Todd, K.S. 1995. Sources and reservoirs of Toxoplasma gondii infection on 47 swine farms in Illinois. J. Parasit., (In press).

Weigel, R.M., Dubey, J.P., Siegel, A.M., Hoefling, D., Reynolds, D., Herr, L., Kitron, U.D., Shen, S.K., Thulliez, P., Fayer, R., and Todd, K.S. 1995. The prevalence of antibodies to Toxoplasma gondii in swine in Illinois in 1992. J. Am. Vet. Med. Assoc. 206:1747-1751, (In press).

TOXOPLASMA GONDII RECOMINANT ANTIGEN

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CRIS Number: 1265-32000-045
Termination Date: March, 1999
FSIS Number: I-94-10

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OBJECTIVE: Develop low-cost assay to detect antibodies to Toxoplasma.

PROGRESS: The objective of this project is to develop a reliable, sensitive, specific and easy to perform assay to detect antibodies to Toxoplasma gondii in naturally infected animals. In order to have reliable antigen available in large quantities, 6 recombinant antigens of T. gondii, H11, H4, B427, V22, C55, and C51, were assessed for application in ELISA tests for toxoplasmosis in swine. The antigens were evaluated with sera from experimentally infected, young pigs and with sera from slaughter sows bioassayed for T. gondii. Recombinant antigen ELISAs demonstrated a sharp rise in response very early after infection when used to test sera from experimentally-infected pigs. In comparison to ELISAs with native antigen, recombinant antigens were more reactive with early post-infection sera, and less reactive with late (chronic) infection sera. For slaughter sows, detection of Toxoplasma gondii in heart tissue by bioassay was the gold standard used to assess ELISA results. ELISAs, using a combination of B427 and V22 antigens, detected 23 of 37 bioassay positive sows (62% sensitivity) with 4 false positive reactions (92.5% specificity). Native antigen ELISA detected 18 of 37 bioassay positive sows (48.6% sensitivity) with 1 false positive (98% specificity). These results are encouraging and efforts are being made to test more recombinant antigens.

PUBLICATIONS

None

DETERMINATION OF THE SAFETY OF CHLORINE OR OTHER DISINFECTANTS USED IN MEAT AND POULTRY PROCESSING

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CRIS Number: 5325-42000-016
Termination Date: June 1997
FSIS Number: I-82-34

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OBJECTIVE A: Investigate systematically the causal relationship between chlorination and mutagen formation in food processing water and determine the specific mutagens formed upon chlorination.

PROGRESS A: Chlorination of poultry chiller water (PCW) by chlorine is an effective process for achieving significant reductions in the levels of bacterial pathogens. However, a few of the chemical by-products of chlorination are of concern because of their potential toxicity. This research addresses the problem of risk analysis by focusing on the molecular identification of trace chemicals which react positively in the Ames bacterial mutagenicity test. Because the Ames test is only an indicator of toxicity, it is important to know which of the thousands of chlorination by-products are responsible for Ames activity; such knowledge puts risk evaluation on a more secure basis. Our recent identification of a compound called a tetrachloro-imide in simulated poultry chiller water was a significant breakthrough chloro-imide suggests it accounts for a large fraction of the mutagenicity in actual PCW. If further studies, now underway, confirm this information, then methods for deactivating the actual processing solutions could very likely be developed. The identified compound is a reactive electrophile, and preliminary studies show that common amino acids may deactivate it to innocuous covalently bound forms. In fact, the broilers themselves may provide a matrix for deactivation. The chloro-imide is chemically similar to the mutagen MX identified in drinking water in 1988. We have begun to explore computer-calculation methods that may establish a unified method to predict the toxicity of chlorinated molecules; preliminary results are that MX and the chloro-imide can be treated with the same types of computations. Model compounds have been synthesized to test these theories.

OBJECTIVE B: Investigate potential disinfectants, other than chlorine, of food processing water.

PROGRESS B: For some time we have felt that chlorine dioxide is a viable alternative to the use of chlorine as a disinfectant in poultry chiller operations. Based in large part on work in this CRIS, in March FDA approved an industry petition filed by Rio Linda Chemical Company for in-plant trials of chlorine dioxide in poultry chiller water (PCW). FDA received ten requests for approval of in-plant trials of chlorine dioxide and work is now underway in several plants nation-wide. There are at least two significant potential benefits to chlorine dioxide compared to chlorine. First, the production of trihalo-methanes (THM's) and Ames-active disinfection by-products appear to be reduced negligible values, and second, chlorine dioxide appears to achieve bacterial reductions using four to five times less chlorine than required when chlorine is used. Work in this

unit showed, furthermore, that the major chemical products of chlorine dioxide, chlorite and chlorate ions, are below the accepted safe levels in the product, and that changes in fatty acid profiles are insignificant. Chlorite/chlorate measurements were carried out in support of the Rio Linda petition using new chromatographic methods developed in our laboratory to replace prior methods, now unacceptable based on amperometic detection. The new method also provides values of free chlorine dioxide. Current work addresses method development for in-plant tests suitable for use by plant personnel.

IMPACT/ TECH TRANSFER: The work on chlorine dioxide acceptability and efficacy has been transferred to industry via approval of the Rio Linda Chemical and the inititation of in-plant trials of chlorine dioxide.

OBJECTIVE C: Filtration studies of processing water and chiller brine.

PROGRESS C: Further tests were made to determine the effects of prefiltration of brine with diatomaceous earth (DE) filters on subsequent microfiltration flux through ceramic "model" brines made in the laboratoary. DE filtration parameters included precoat levels, bodyfeed rates, and filter media. Regardless of the combination of parameters used for DE the effects on subsequent microfiltration rates were about the same. The DE prefiltration resulted in very high initial flux in subsequent microfiltration, but after about 30 minutes the microfiltration flux decayed to nearly the same level brines that had not been DE prefiltered. Results were similar for the plant and model brines. For both plant and model brines preheating to about 80 °C prior to microfiltration resulted in substantial flux increases that were sustained. Although DE filtration did not result in sustained increased in microfiltration flux, using about 0.25-0.5% total DE, equally divided between precoat and bodyfeed, and a DE impregnated filter media, resulted in substantial microbial reductions in the brine. This result indicated that DE filtration alone, without the use of subsequent microfiltration may be sufficient to greatly extend brine use. DE filtration trials were made on chiller brine at a bacon processing plant site using the same apparatus that was used for the pilot tests. The plant disposes of brine after one-day use in accordance with guidelines. For these tests, samples of the brine were DE filtered prior to disposal. Results indicated that DE filtration reduced the standard plate counts from about 1000/ml to about 10/ml, and total coliform and fecal coliform counts were each reduced to <2.2/100 ml. As a result of these tests, plant personnel further tested the filtration equipment to assess its potential for use in their system.

IMPACT/TECH TRANSFER: Through a joint effort with the processor and the equipment supplier, a Partial Quality Control Program (PQCP) was prepared and submitted to FSIS. Because of a change in FSIS procedures, the PQCP is being revised for submission to FSIS as a Research/Technology Assessment Protocol.

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CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON POULTRY CARCASSES WITHIN THE PROCESSING PLANT

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OBJECTIVE A: Pre-evisceration treatments; evaluation of spray scalder.

PROGRESS A: Measurements of scald water temperature at different spray nozzles were taken and adjustments were made to nozzles accordingly to improve heat control of the spray. After adjusting the nozzles, carcasses were spray scalded and picked in a single pass picker to determine the optimum picker parameters. Results of the spray scalding and picking trials will be compared to the standard immersion scald (34 °C for 2 min) and pick. Thermocouples were inserted beneath the skin and on top of the feathers of carcasses to measure temperatures and determine heat penetration to the muscle tissue. Future work will include electron microscopy of skin samples to determine the effect of heat on the skin and feather follicles in conjunction with microbiological trials comparing immersion scalding to spray scalding.

IMPACT/TECH TRANSFER: Spray scalding research continues under a CRADA with Johnson Food Equipment, Kansas City, MO.

OBJECTIVE B: Test computer simulations on the fate of bacteria suspended in scald tanks.

PROGRESS B: A scale model scald tank was constructed to simulate commercial scalding conditions. Water in the tank was mixed with compressed air. Food coloring was added at a single point and water samples were taken periodically at various distances from the point of addition. An effective mixing cell size was determined for several tank configurations. Results confirmed that a previously developed computer model can predict mixing patterns of particles or bacteria suspended in water.

OBJECTIVE C: Pre-evisceration treatment of carcasses; GRAS chemical spray during pick.

PROGRESS C: Spray nozzles were installed in a pilot plant picker and a metering system fabricated for spraying a 1% acetic acid solution on the feathered carcasses during the defeathering process. Sample carcasses were picked while being continuously sprayed with either water or a 1% acetic acid solution during a 30 sec picking time. Acetic acid treatment of carcasses during picking resulted in a 0.6 $\log_{10}$ CFU/ml reduction in total aerobic plate counts when compared to the water sprayed control carcasses.

IMPACT/TECH TRANSFER: The picking process is a major cause of cross-contamination among carcasses. The addition of GRAS chemicals to the picker should reduce microbial counts and the incidence of enteric pathogens exiting the picker.

OBJECTIVE D: Testing the effect of hand-brushing on microbiological quality of carcasses.

PROGRESS D: New York dressed carcasses were scrubbed by hand-brushing with (2%v/v) solutions of acetic acid and tri-sodium phosphate (TSP) in the pilot plant laboratory. Temperature of the acid, TSP solutions and tap water applied to the carcasses was approximately 15 C (60 °F). Carcass treatments were: (1) spray washed controls; (2) hand-brushed carcasses with tap water; (3) hand-brushed carcasses with TSP; (4) hand-brushed carcasses with acetic acid solution. Aerobic and Enterobacteriaceae counts were determined by the whole carcass rinse. Phosphate buffered saline (.1N) was used to neutralize the acid and TSP in the whole carcass rinse procedure (pH 7.6 and 6.8 respectively). No statistical differences were detected when Enterobacteriaceae and aerobic bacteria counts for the treatment carcasses were compared to the controls.

OBJECTIVE E: Electrical stimulation during bleeding and defecation patterns.

PROGRESS E: It has been reported that electrical stimulation of poultry carcasses during bleed out elicits about 87% of the carcasses to defecate. Installation of stimulation equipment in commercial operations (with early deboning of breast meat as the primary objective) has supposedly resulted in a decrease in the number of carcasses reprocessed due to fecal contamination. The decrease has been noted by processing plant quality assurance personnel but data is not available to substantiate the findings at this time.

IMPACT/TECH TRANSFER: Presently three processors are either using electrical stimulation during bleed-out or are in the process of installing the equipment. From a food safety standpoint, the use of stimulation during bleeding may contribute to lower numbers of reprocessed carcasses because enteric bacteria are confined in the initial stages of processing (scald tank), thus lowering the possibility of cross-contamination during the evisceration process.

OBJECTIVE F: Processing plant air quality and methods of sampling the air.

PROGRESS F: Poultry processing air was sampled to provide a profile of the air borne microorganisms in the defeathering and evisceration areas. A standard impinger and single stage impactor air sampler were evaluated to determine their efficiency for collecting and enumerating microorganisms in the air inside the processing plant. With the standard impinger, it was possible to detect very low numbers of microorganisms. The impactor was not as sensitive as the impinger. Using a single sample from one sampling period, the standard impinger can be used to enumerate a number of different kinds of microorganisms, but the impactor is limited to microorganisms that can be recovered on a single media. The air samples were evaluated to determine levels of molds, aerobic, Enterobateriaceae, Salmonella, Campylobacter, E. coli, and Staphylococcus present in the defeathering and evisceration areas of a commercial processing plant. The $\log_{10}$ aerobic counts per 1,000 liters of air with the impinger and the impactor were 7.77 and 6.49, respectively. Enterobacteriaceae (6.49) and Staphylococcus (7.45) could only be detected with the impinger, while molds and fungi (2.59) were detected only with the impactor. No Salmonella, Campylobacter, or E. coli were detected with either sampling procedure.

OBJECTIVE G: Study formation and composition of biofilms on processing plant surfaces and on poultry skin and meat.

PROGRESS G: Initiated baseline studies to describe the formation of biofilms and the portent of pathogens within the biofilms in the processing plant environment. Five trials have been undertaken for spectrophotometric and electron microscopy studies of bacterial attachment to various processing plant surfaces. Samples of whole carcass rinses and

multiple chicken tissues were collected from a commercial, broiler processing plant after the New York rinse. Carcass rinse solutions were measured by spectrophotometry after exposure to an array of surface samples in solution and compared with control solutions at hourly time periods at each of a series of dilutions. Stainless steel, polyethylene, belt webbing and broth controls had approximately the same attachment activity. Bacterial attachment to rubber picker finger material appears to exceed that of attachment to other surfaces. Data from these and subsequent studies will provide important information on the components of bacterial attachment and the relative participation of pathogens and non-pathogens in the contamination of broiler carcasses and processing plant surfaces.

IMPACT/TECHNOLOGY TRANSFER: Application of this information can lead to the reduction of contamination in the processing environment and the improvement of process sanitation.

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CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON RED MEAT

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OBJECTIVE A: Determine the efficacy of various antimicrobial compounds for use in decontaminating red meat animal carcasses.

PROGRESS A: The efficacy of chlorine dioxide (ClO_2) to reduce bacterial contamination on fecally contaminated beef was examined. Beef carcass surface tissues (BCT) were inoculated with bovine feces and spray treated (10 s; 75 psi; 16 °C) with ClO_2 (20 ppm). Bacterial populations were reduced by < = 0.93 log CFU/cm2, regardless of ClO_2 concentration, and were not statistically different (p > = 0.05) than water treatments. In a subsequent study, tap water (16 °C) and ClO_2 (20 ppm) were sprayed (100 psi) for 15, 30, and 60 s onto inoculated BCT. Reductions (1.53 to 2.07 log CFU/cm^2) from ClO_2 were not statistically different (p > = 0.05) than water treatments. These results demonstrate that spray treatments with ClO_2 are no more effective than water for reducing fecal contamination on beef. Copper sulfate ($CuSO_4$) spray washes were studied as a means of reducing fecal contamination on beef carcasses. Inoculated beef carcass tissue was spray treated with 20, 40, 80, and 160 ppm $CuSO_4$ for 10 seconds. Even at 160 ppm, spray washing with $CuSO_4$ was no more effective than water for reducing bacteria. Subsequently, the residual effect was determined by treating samples (as described) and testing after 0, 24, and 48 hours. Overall, bacterial populations remaining after spray treatments with $CuSO_4$ were no different than water spray treatments. However, reductions of bacterial populations after 48 hours were greater (0.30 log bacteria/square centimeter) with spray treatments at 160 ppm as compared to water or lower concentrations of $CuSO_4$.

IMPACT/TECH TRANSFER A: This research demonstrates that the use of said concentrations of chlorine dioxide or copper sulfate offers no advantage over water spray treatments to reduce bacterial contamination of beef carcass surfaces.

OBJECTIVE B: Determine the efficacy of using bacteriocins as carcass decontaminating agents to inhibit Salmonella typhimurium and E. coli O157:H7.

PROGRESS B: Salmonella typhimurium and E. coli O157:H7 were treated in vitro with combinations of nisin and food additives (EDTA, sodium hexametaphosphate, citric acid, and lactic acid) at 98°F or 41°F to determine what factors (temperature, interfering compounds) affected bacterial reductions. Combinations of food additives and nisin killed up to 62% of S. typhimurium and up to 70% of E. coli O157:H7 at either temperature. In a subsequent study, beef tissue inoculated with E. coli O157:H7 and S. typhimurium was treated with combinations of nisin and food additives (EDTA, sodium hexametaphosphate, citric acid, and lactic acid) and stored at 41 °F for up to three days. Nisin and additive combinations reduced the pathogens less than 6%.

IMPACT/TECH TRANSFER B: The use of nisin alone or with food additives to inhibit gram negative pathogens on meat is not efficacious.

OBJECTIVE C: Determine the efficacy of using bacteriocins as carcass decontaminating agents to inhibit gram positive spoilage bacteria and listeriae.

PROGRESS C: Nisin spray treatments were examined as a means of reducing gram positive bacterial populations on beef carcass tissues. Beef carcass surface tissue was inoculated with Brochothrix thermosphacta (BT) or Listeria innocua (LI) and subjected to spray treatments with sterile water or nisin (5000 AU/ml). Samples were vacuum packaged and incubated at 4 °C and enumerated at 0, 7, 14, 21, or 28 days. Nisin spray treatments of lean and adipose vacuum packaged tissues reduced LI up to 2.83 log CFU/cm^2. Additionally, nisin sprays and vacuum packaging effectively suppressed LI during the 4-week incubation such that the remaining bacteria did not grow to the same level as untreated or water treated, vacuum packaged tissues. Nisin spray treatments and vacuum packaging of lean and adipose tissues reduced BT to undetectable levels.

IMPACT/TECH TRANSFER C: This study demonstrates that a multi-hurdle approach involving nisin spray treatments followed by vacuum packaging under refrigerated conditions reduced spoilage and model pathogenic bacteria on carcass surfaces.

OBJECTIVE D: Determine the efficacy of an edible film or carrier as a delivery system for enhancing the antimicrobial effectiveness of bacteriocins in or on meats.

PROGRESS D: Sections of U.V. sterilized lean and adipose beef carcass were inoculated with Brochothrix thermosphacta (BT), a meat spoilage organism. Samples were left untreated (U), treated with calcium alginate (A), nisin (N), or nisin immobilized in calcium alginate (AN). Samples were held at 4 °C and sampled for bacterial populations and nisin activity at days 0, 1, 2, and 7. U and A treatments of lean and adipose beef did not affect bacterial growth, whereas N and AN treatments suppressed bacterial growth. Preliminary data indicate that nisin and alginate treatments were more effective for reducing bacterial populations than nisin treatments alone. Titers of bacteriocin activity were greater from samples treated with AN than N. These data suggest that immobilization of nisin by calcium alginate may be a more effective delivery system of bacteriocin to the carcass surface than a liquid rinse or spray.

IMPACT/TECH TRANSFER D: This study reports immobilization of a bacteriocin by calcium alginate for reducing bacterial populations on beef. The information obtained from this study will be used in subsequent experiments in which immobilized bacteriocins (brochocin-C, colicins) are incorporated into a ground product for reducing L. monocytogenes and E. coli O157:H7.

OBJECTIVE E: Determine the effectiveness of hot water washes for removing bacterial fecal populations from beef carcasses.

PROGRESS E: Two separate studies were conducted to determine the effectiveness of various temperature water spray washes (Wt) and wash/steam combinations (WtS) for reducing fecal bacteria on sheep and beef carcasses. Wt of 15.6, 54.4, and 82.2 °C were administered to sheep carcasses contaminated with feces, using a hand-held spray nozzle. Initial carcass bacterial populations of approximately 2.5, 4, and 6 log CFU/cm2 were subjected to all wash combinations. W82.2 and W82.2S reduced bacterial populations as much as 4.0 log cycles. The initial contamination levels (4 and 6 log cycles) had little effect on final bacterial levels (2.7-3.3 log cycles) after carcasses were subjected to WtS and W82.2. However, uninoculated carcasses with initial bacterial populations of 2.5 log CFU/cm^2, experienced a 1.5 log cycle reduction after subjected to WtS and W82.2. It is possible that hydration of a carcass before and during interventions affords some protection to bacteria. The second study used a commercial carcass washer to apply a hot water (72 °C), low pressure (20 psi) wash in combination with a high pressure (125 psi), warm water (30 °C) wash (W72/30). Reductions on beef of 2.7, 3.3, and

3.4 log cycles for APC, coliforms and E. coli populations, respectively, were observed. Implementation of a moist heat either with a spray wash or steam would increase the microbial safety of beef carcasses.

IMPACT/TECH TRANSFER E: The use of an antimicrobial wash of some type has been mandated to the beef industry by FSIS. Consequently, various beef processing plants as well as FSIS have the need to know the effectiveness of hot water washes on beef carcasses. The FSIS has been informed of these results through summary reports. Processing corporations as IBP, Beef America, Cargill, ConAgra, Monfort, and Lovett & Sons have received the results of this work through direct contact. Commercial carcass washing engineering companies (Carey Engineering and Chad Engineering) have also received the information.

OBJECTIVE F: Determine the effectiveness of a steam-vacuuming system alone and in combination with a commercial hot water wash for removing bacterial fecal populations and E. coli O157:H7 from beef carcasses.

PROGRESS F: A commercial steam-vacuum used in conjunction with a commercial combination wash of hot water (72 °C) at low pressure (20 psi) in combination with a high pressure (125 psi), warm water (30 °C) wash (W72/30), reduced bacterial levels on contaminated beef carcass short plates by 3.1, 4.2, and 4.3 log cycles for APC, coliforms and E. coli populations, respectively. A second study using the steam-vacuum sanitizer reduced aerobic plate counts associated with bovine fecal contamination from 5.5 log CFU/cm^2, to 3.0 log CFU/cm^2 on beef carcass short plates. The same beef carcass short plates in this second study inoculated with 7.6 log CFU/cm2 E. coli O157:H7 in feces, yielded an average residual level of E. coli O157:H7 of 2.1 log CFU/cm2, after steam-vacuum treatments. This study demonstrates the effectiveness of a steam-vacuum sanitizer for removing E. coli O157:H7 from beef carcasses.

IMPACT/TECH TRANSFER F: These two studies indicate the use of a steam vacuum system in combination with a commercial water wash could reduce the amount of trimming needed on carcass processing lines. The use of new and innovative heat intervention technologies is important to beef processors as well as FSIS under the zero tolerance rule. Consequently, various beef processing plants as well as FSIS have a need to have any new technologies effectiveness quantified. A steam-vacuum system could potentially benefit the industry economically by avoiding previously wasted product as well as improve the microbial safety of beef carcasses. The FSIS has been informed of these results through summary reports. Processing corporations as IBP, Beef America, Cargill, ConAgra, Monfort, and Lovett & Sons have received the results of this work through direct contact. The company that developed this equipment, as well as others, have also received the information.

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CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON RED MEAT

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OBJECTIVE A: Develop and evaluate a means to rapidly detect microbial and accidental fecal contamination on animal carcasses; to use this method as a HACCP monitor and as a test for reconditioned carcasses.

PROGRESS A: FY 93: A rapid microbial ATP bioluminescence test (R-mATP) was developed in FY 93 that will detect high levels of generic bacteria (indicative of fecal contamination) on carcasses. FY 94: The R-mATP was validated for beef, pork and poultry. (for R-mATP test details refer to 1993 and 1994 Annual Reports). FY 95: The R-mATP test was further tested in poultry, beef and lamb plants. Data requested by the FSIS was obtained and submitted to the FSIS ATP/BIT committee. This data includes the following information: Conversion charts were developed to convert raw data to log APC equivalent. Commercially available ATP and lyophilized bacterial controls were evaluated. Preliminary and brief data on ruggedness were obtained. Data relating total generic bacterial counts with coliforms and generic E. coli was obtained from a sampling of 620 beef carcasses. Regression lines of scatterplots (log APC/cm^2 vs. log R-mATP test result/cm^2) indicate that between plants within species (three beef plants and three poultry plants) the response curves were not significantly different. This assay demonstrates a similar test response between plants as well as the possibility of using one large and representative database from within one species for several similar processing plants. Data collected from poultry plants (n = 120) indicate that the R-mATP can successfully discern samples that are > = 4 q 0.75 log APC/ml of poultry carcass sponge samples 92% of the time.

IMPACT/TECH TRANSFER A: The R-mATP assay has been validated as a rapid indicator of high levels of microbial contamination for beef, pork, and poultry. This assay can be used to monitor critical control points in animal processing such as after evisceration in the case of beef and pork and immediately prior to chilling in the case of poultry processing. The FSIS ATP/BIT committee continues to evaluate the R-mATP as a rapid HACCP monitor. Industrial interest continues with actual trial testing having been done in Nebraska (Beef America, Norfolk, NE) and California (Superior Packing, Dixon, CA). Other potential user groups include the Australian Meat Research Corporation as well as Tepnel Life Sciences of the United Kingdom.

OBJECTIVE B: To determine the effectiveness of several rapid and nondestructive carcass sampling methods.

PROGRESS B: Six bacterial sampling methods that might be used for rapid sampling of beef carcasses were evaluated in 2 separate studies. In Study 1, bacterial recovery from uninoculated beef rounds was 2.6, 2.3, 2.1, and 1.3 log CFU/cm^2, respectively, for excision (EX), and swabbing with Cheesecloth (CC), Sponge (SP), and cotton tipped wooden swabs (CS). For Study 2, beef tissue was inoculated with bovine feces at different levels and the mean recovery was 3.7, 3.0, 3.1, and 3.1 log CFU/cm^2, respectively, for EX, and swabbing with SP, Griddle Screen (GS), and 3M Mesh (M). For both studies, EX was determined to be the most consistently effective method while the initial study determined swabbing

with CS was the least effective of the methods used. In both studies, the most abrasive materials approached the effectiveness of EX even at low inoculation levels. As the inoculation levels increased, the additional effect of abrasiveness was lessened. When the carcasses were contaminated with bovine feces, the bacterial populations that were rapidly recoverable from beef tissue using SP, GS, or M were not significantly lower than those recovered using EX. Consequently, SP, GS, or M are an adequate method of beef carcass sampling for rapid, in-plant process monitoring to detect fecal contamination.

IMPACT/TECH TRANSFER B: Excision is the most effective method for sampling beef carcasses. However, excision sampling is not rapid nor easily reproducible between samplers. As a result, it is unlikely that excision will ever be a practical rapid sampling method for a processing plant quality control monitoring program that is attempting to collect samples for rapid microbial tests from a moving processing line. The validation of rapid and nondestructive sampling methods will aid meat processors in determining sampling methods for rapid, in-plant microbial monitoring. Sponge sampling can be used for a number of rapid microbial tests.

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PREVENT THE ATTACHMENT OF FOODBORNE PATHOGENS TO POULTRY SKIN AND MUCUS

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OBJECTIVE A: Determine mechanism and site of Salmonella attachment to poultry skin

PROGRESS A: Modification of poultry skin surfaces by treatment with formalin (modifies amino and carboxyl groups in proteins), papain (hydrolyzes peptide and ester bonds) , lipase (hydrolyzes lipids) and trisodium phosphate (removes unidentified organic materials) had no effect on the subsequent attachment level of Salmonella. This suggests that modifications of poultry skin without significant structural alterations will not influence Salmonella attachment. The bacterial cell concentration affected the proportion of the cell population that irreversibly attached to chicken skin after 1 h as compared to total numbers on skin, including cells in the surface water film. At 107 Salmonella cells/ml and 108 cells/ml inoculation level, irreversibly attached cells accounted for 93 % and 38 %, respectively of the total surface cell population. This suggests that the low numbers of salmonellae cells typically found on poultry will become strongly attached over a period of time.

OBJECTIVE B: To determine the position of attached or entrapped Salmonella cells in poultry skin.

PROGRESS B: Using the optical sectioning feature of the Confocal Scanning Laser Microscope, external and internal 3-D images of poultry skin were obtained without artifacts associated with dehydration and other sample preparation techniques. Images of poultry skin exposed to Salmonella cells indicated that the cells associated with the skin were mostly located in the crevices or feather follicles of poultry skin. Many cells were not attached but in the water film Results suggest that strategies or modifications to prevent entrapment of water or that change microtopography of skin may be helpful in reducing contamination of skin.

OBJECTIVE C: To determine the mechanism of attachment of Salmonella to chicken intestinal mucus and identify intestinal isolates of indigenous bacteria that block attachment.

PROGRESS C: Attachment of S. typhimurium cells to cecal mucus in the presence of D-mannose (to block mannose-sensitive attachment mediated by type 1 fimbriae) was enhanced by the presence of calcium and inhibited by chelating agents, indicating that divalent metal cations play an important role in attachment. The attachment of S. typhimurium to mucus was also inhibited by pre-exposure of mucus to spent culture supernatant and/or washed cells of some of the lactobacilli strains isolated from chicken ceca and crops. The factor(s) that inhibited attachment were non-dialyzable. Attachment of S. typhimurium to mucus (in the absence of mannose) was inhibited by pre-exposure of mucus to some of the strains of Enterobacteriaceae isolated from ceca and crops. This form of attachment was also inhibited by type 1 fimbriae isolated from these Enterobacteriaceae strains. Trials are underway to determine if giving to young chicks strains of intestinal bacteria that inhibit attachment to intestinal components reduces intestinal colonization of salmonellae.

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MICROBIAL SAFETY CRITERIA FOR FOODS CONTACTING REUSE WATER IN FOOD PLANTS

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OBJECTIVE A: To study the potential for expanded applications of reuse water in the food plant environment.

PROGRESS A: Truck Washing to Reduce the Incidence of Salmonella. The efficacy of washing pig hauling trucks to break the Salmonella cycle was evaluated. The premise is that cleaning and sanitizing the trucks between pick-ups should eliminate/reduce the incidence of Salmonella brought to the second farm. The factors investigated which could influence the incidence and level were: a) distance traveled 'short haul' (<500 miles) vs. 'long haul' (>500 miles); b) season of the year; c) full vs. part load; and d) geographic site of the farm. Though additional trucks need to be sampled to ensure a valid statistical design, preliminary assessment of the data indicates that 1) short haul trucks were less contaminated than long haul trucks and 2) washing the trucks (first rinsed with either potable or reconditioned water) with an alkaline detergent followed by treatment with a sanitizer dramatically reduced the incidence and level (MPN) of both Salmonella and coliforms. As part of this study, the sensitivity of certain rapid methods (Tecra and a PCR method developed by DuPont) were compared to the standard FDA/FSIS cultural MPN method for Salmonella. At present, Tecra is similar to the MPN procedures in that Tecra can predict low levels of Salmonella; however, the sensitivity of the PCR method must be increased to make it a useful technique for the detection of low levels of Salmonella. We are also cooperating with the National Animal Disease Center, Ames, Iowa and DuPont to serotype and ribogroup the Salmonella strains isolated from the trucks. When completed, this will provide epidemiological information of the seasonal incidence and geographic distribution of specific sero- and ribotypes of Salmonella and thus permit development of distribution and transmission patterns.

Pathogen Growth in Reconditioned Water. The growth and/or survival potential of various food-borne pathogens in reconditioned pork processing plant water held at different temperatures was investigated. The pathogens studied included both Gram positive (Streptococcus faecium, Staphylococcus aureus, and Listeria monocytogenes) and Gram negative (Aeromonas hydrophila, Salmonella, and Vibrio cholerae) bacteria. For some reconditioned water samples, sodium thiosulfate was added to neutralize residual chlorine. The Gram positive bacteria did not grow, but remained viable in reconditioned water with the chlorine neutralized, especially at low holding temperatures In contrast to the Gram positive bacteria, the Gram negative bacteria grew. The three Gram negative bacteria generally increased in numbers by ca 3 log units. In addition, all three were inactivated by the residual chlorine in the reconditioned water and died off unless the chlorine was neutralized by the addition of 10 mg/L sodium thiosulfate. The specific responses were a function of individual bacteria. A. hydrophila increased in number from 10^3 to 10^6 cfu/ml at temperatures ranging from 5 to 42 °C and remained at that level for extended periods (up to 80 days), particularly at temperatures of 28 °C and below. The nutrient content of the reconditioned water was determined by the coliform growth response (CGR), a bioassay system which uses a specific culture of Enterobacter cloacae. The CGR of the reconditioned water used in these studies was 2.91+/- 0.64, which agrees

with the 3 log units of growth of A. hydrophila observed in these studies. Based on these studies, care must be exercised in maintaining the residual chlorine levels of the water and not recontaminating it with Gram negative pathogens. Using a temperature gradient incubator, Salmonella and V. cholerae strains were tested for the growth and/or survival potential over a temperature range of 4 to 50 °C in filter-sterilized unchlorinated reconditioned (fur) water. The mixture of Salmonella strains grew in the fur water at temperatures of 12 to 40 °C; below 12 °C, the count remained at the starting level, while at temperatures above 40 °C, the number of viable Salmonella declined. The maximum increase in numbers occurred at temperatures from 20 to 37 °C. The mixture of V. cholerae strains grew between 10 and 37 °C; below 10 °C and above 37 °C, there was no increase in cfu/ml, but the strains survived. As with A. hydrophila, both Salmonella and V. cholerae grew in the reconditioned water in proportion to the CGR of the water employed.

OBJECTIVE B: To investigate new methods to determine food product safety, water safety, and sanitation quality.

PROGRESS B: F+RNA coliphage. Research continued to assess the efficacy of using E. coli bacteriophages as an indicator of water quality in specific commercial unit operations of pork slaughter. Phage is more chlorine resistant than most bacterial indicators, and the plaque assay results are available 18 h after plating. Water from the scald tank, dehairing machine, and polishers were evaluated to compare coliphage values with total aerobic plate counts and coliform assays using 3M petrifilm. Generally, there was a high correlation between phage and coliform assays. Coliphage assays were also performed in parallel with bacteriological assays in the truck washing study, described elsewhere. Results will be described in a future report. It is anticipated that this research will be completed during FY 1996.

OBJECTIVE C: To identify means to prevent microbiological hazards that are barriers to the increased utilization of reuse water during slaughter and further processings and establish safety criteria.

PROGRESS C: Solution Reuse. The study was completed on growth, injury, and survival of Yersinia enterocolitica, Listeria monocytogenes, and Staphylococcus aureus in a model propylene glycol (PG) chilling system. Temperatures studied varied from 28 to -12 °C and PG levels were from 0-60%. Sampling duration was up to 30 days. Y. enterocolitica grew r 1 log in < 200 hours at temperatures r 5 °C and at a PG concentration of s 8%. No death was detected at temperatures below -2 °C in 30 days. L. monocytogenes grew r 1 log in < 200 hours at temperatures r 12 °C. and at PG levels s 8%. L. monocytogenes grew r 1 log in < 300 hour at temperatures r 5 °C. and PG levels s 8%. No death was reported at temperatures below -2 °C. in 30 days. S. aureus did not grow below 28 °C and at PG levels > 8%. Death was recorded for S. aureus at temperatures below -2 °C. No significant injury was reported for any of the organisms tested. The results demonstrate that growth of these pathogens can be controlled in cooling solutions. However, their prolonged survival indicates a potential risk if pathogens are transferred to the surface of the food being cooled.

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SCREENING TEST FOR HAPTOGLOBIN

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OBJECTIVE A: Develop an Immunological-based test for screening cattle at slaughter for the presence of the acute phase reactant, haptoglobin.

PROGRESS A: An immunoassay has being developed to detect the presence of haptoglobin, an acute phase reactant, in the serum of cattle. Detection of acute-phase reactants such as haptoglobin have been identified by FSIS as a priority item in the development of better methods for antemortem evaluation of beef cattle prior to slaughter. We have generated a monoclonal antibody to haptoglobin following immunization with a partially purified immunogen. Hemoglobin binding to the haptoglobin does not interfere with binding of our monoclonal antibodies. We had previously demonstrated three different immunoassays for determining levels of haptoglobin in bovine serum samples. These three methods are (i) Direct Sera Binding in Microtiter Plates, (ii) A Direct Hemoglobin Binding Immunoassay, and (iii) A Haptoglobin Competitive Inhibition Immunoassay. These three methods were verified in a collaborative study with an FSIS scientist (Dr. P.K. Saini) using slaughterhouse serum samples. We have assayed over 2,000 bovine samples involving some 30,000 assays using the most reliable and reproducible of the three immunoassay formats, the Direct Hemoglobin Binding ELISA.

IMPACT/TECH TRANSFER: The following studies have been completed and are in various stages of publication. A patent application was submitted and has been allowed by the patent office.

1. Evaluation of haptoglobin levels in feed lot cattle with respiratory disease and study of the effects of antibiotic treatment on haptoglobin levels. A collaborative study with ARS scientists Dr. Travis Littledike and Dr. Tom Wittum, USDA-ARS, Clay Center, NE.

2. Evaluation of haptoglobin levels in bob veal calves with Icteric fever. A collaborative study with FSIS scientist Dr. Mary Gray, University of Georgia, Athens,GA.

3. Evaluation of haptoglobin levels in cattle with Lyme disease and with Johne's disease or with both diseases. A collaborative study with university scientist Dr. Tom Collins, University of Wisconsin, Madison, WI.

4. Evaluation of haptoglobin levels in cattle with Milk fever. A collaborative study with ARS scientist Dr. Jesse Goff, USDA-ARS, Ames, IA.

5. Evaluation of haptoglobin levels in cows with mastitis undergoing antibiotic treatment with either penicillin or with ceftiofur. This is an active collaborative study with Dr. William Smith, University of Florida.

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SPECTRAL RADIOMETRY AS ON-LINE INSPECTION TOOL FOR POULTRY

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OBJECTIVES: (1) Collect multispectral images and visible/near-infrared (Vis/NIR) spectral data of the viscera and the inside and outside surfaces of poultry carcasses of normal, septicemic, cadaver, bruised, tumorous, air-sacculitic, and ascites carcasses on-site at various slaughter plants to establish a complete data base. (2) Develop classifiers for on-line classification of wholesome and unwholesome poultry carcasses, using a multispectral imaging and a Vis/NIR spectroscopic techniques. (3) Evaluate methods to implement both systems on-line at poultry slaughter plants and analyze the economic feasibility of implementing a real-time, on-line automatic inspection system.

PROGRESS: Our study showed that a Vis/NIR system, with the probe in a housing, can completely separate the bruised, air sacculitis and ascites birds from the wholesome birds, and it also can separate the cadaver and septicemic birds with an error less than 3%. This means that we can have an inspection system with the percent of bad birds (septicemic, cadaver, bruised, ascites, or air-sacculitis) classified as wholesome and the percent of the wholesome birds classified as bad birds to be less than 3%.

We found that a Vis/NIR system, without the probe touching the bird, with a sensing speed as short as 0.6 seconds/bird (on a moving shackle), and operated in ambient light, can also accurately classify bad and wholesome birds. We found that it does not make any difference whether the birds are moving or not. The distance of the probe from the carcass is the primary cause of error, but it can be mathematically corrected. We concluded that it is possible to make the measurement in under 1.0 second, in ambient light, with the chicken moving (60 birds/min), without the probe touching the bird, and without controlling the distance too carefully, although we may have to measure the distance.

We studied the feasibility of using multispectral cameras for inspection. We found that they could perfectly separate the septicemic and cadaver birds from the pass-inspection line, and less than 10.7% of the wholesome birds would be in the rejected line. The error for separating tumorous birds from the wholesome birds was 8.6%. Due to the small number of samples available, we have no definitive results on the separation of bruised and skin-tear birds from the wholesome birds yet, but excellent separation is expected, because the Fourier spectra of the bruised and skin-torn birds were very much different from those of the normal.

A scaled-down processing line equipped with an on-line inspection system is being assembled for use off main processing line at slaughter plants. The system can be used to collect Vis/NIR signals and images of poultry birds at various wavelengths. With this system, the Vis/NIR probe will be stationary and will not touch the bird. The sensing speed will be as low as 0.32 seconds per bird. The shackle will move at a speed of more than 60 birds per minute. We recently installed a pilot processing system in the Instrumentation and Sensing Laboratory. The system is similar to processing lines at slaughter plants. It can take up to 62 birds and move at a speed of more than 60 birds per minute. An automated inspection system is being set up. The automation system will

include a mechanism which will remove the birds from the processing line, when they are determined to be bad by the instrumental inspection system.

PLANS: We are planning to obtain reflectance spectra of normal and abnormal chicken carcasses (septicemia, cadaver, air-sacculitis, bruise, ascites, tumor) with an experimental apparatus which is equipped with a moving shackle (driven by a motor) and a transportable diode array spectrophotometer at slaughter plants. The experiments will be conducted in room light and off the plant processing line. The experimental system is equipped with a moving shackle (driven by a motor) to simulate the chicken carcass moving along the processing lines. The carcass hung on at the shackle will move to and fro across the apparatus at various processing speeds. The NIR probe will be stationary and not touching the carcasses. The sensing time will be as low as 0.32 seconds per bird. Optical sensors will be used to trigger the sensing of the Vis/NIR system.

In the next few months, we should have an on-line, in-plant inspection system at a slaughter plant (site is being determined) using imaging and Vis/NIR technologies. Design of the system using imaging technology is completed and equipment and parts are being ordered. The on-line, in-plant inspection system will use four black/white cameras with two different wavelength filters. These cameras will be used to image the front and the back of the birds on the moving shackle at the processing line speed. Optical sensors will be used to trigger the capture of the images. An optical line sensor will be used to determine the contour of the chicken breast and will be used to position the birds, if necessary. A computer monitor will display the image of the carcass and different algorithms will be implemented for classifying the carcass.

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MICROBIAL MODELING

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OBJECTIVE A: Develop models (both growth kinetics and inactivation) to estimate the behavior of food-borne pathogens.

PROGRESS A: The current version of the software program containing the growth, survival, and clostridial models, Pathogen Modeling Program version 4.0, continues to be distributed (over 800 copies). We have established a CRADA with a software developer (Simusoft, Inc., Potomac, MD) to rewrite the Pathogen Modeling Program to contain additional features and be "Windows compatible." The company will continuously update the program as new models are developed and will be responsible for marketing and distributing the program. Negotiations continue on the development of an "international database" for the purposes of generating enhanced models and for commercializing a joint software package. A MOU is currently being reviewed by the U.S. and U.K. governments. The U.S. negotiating team has representatives from ARS, FSIS, and FDA.
IMPACT/TECH TRANSFER: This will create a single, worldwide modeling program that will be more comprehensive than either the U.S. or U.K. programs by themselves. It will facilitate cooperation and communication between regulatory agencies in different countries and with companies over the creation of HACCP programs and evaluation of different foods in international trade.

Growth Kinetics Modeling

OBJECTIVE B: Assess the feasibility of developing a means of modeling the impact of microbial competition on the growth of foodborne pathogens in food products.

PROGRESS B: The effect of microbial competition on the growth of Listeria monocytogenes Scott A is being evaluated by determining its growth kinetics when grown in co-culture with other psychrotrophic bacteria, Carnobacterium piscicola LK5, Carnobacterium piscicola 2562, and Pseudomonas fluorescens. The two strains of C. piscicola had a similar effect on L. monocytogenes despite their different bacteriocins. The primary impact on the pathogen was a suppression of the maximum population density (MPD). Generally, lag phase durations and generation times were not affected. Extensive biochemical tests suggested the suppression resulted from consumption of a key limiting nutrient. Similar data for Pseudomonas fluorescens suppression of L. monocytogenes was collected and is being analyzed. **IMPACT/TECH TRANSFER.** The interactions between competitive/spoilage flora and the pathogens have not been extensively studied. The finding that the lag phase and generation times are not affected means that the predictions made by the existing models are not in error because of the indigenous flora.

OBJECTIVE C: Assess the ability of growth kinetic models based on the use of sodium chloride for modification of water activity (a_w) to accurately predict the growth kinetics of pathogenic food-borne bacteria in food systems using other humectants.

PROGRESS C: Humectant Identity: The effect of humectant identity on bacterial growth kinetics is being studied using Escherichia coli O157:H7, a food-borne pathogen for

which growth kinetics models have been successfully developed using sodium chloride as the humectant. Humectants were evaluated include mannitol, sorbitol and sucrose. Other variables being considered are incubation temperature and initial pH. Data acquisition is complete and analysis is underway. Initial analysis of the data suggest that while distinct differences in the response of E. coli O157:H7 can be attributed to humectant identity, these differences are largely limited to the lower a_w levels. At higher a_w levels (a_w greater than or equal to 0.973, w 5% NaCl), the growth kinetics of the pathogen could be reasonably predicted using the previously developed, NaCl-based growth kinetics models. Sequestrants: Development is underway of a response surface model for the effect of a long chain sodium polyphosphate (average chain length, P = 13) on the aerobic growth of L. monocytogenes as a function of temperature (4 to 19 °C), pH (5 to 7) and sodium chloride (0.05 to 4.5%). Data is collected and statistical analysis is underway. **IMPACT/TECH TRANSFER.** This research expands the applicability of the modeling programs to a broader range of foods with reduced water activity.

OBJECTIVE D: Develop and validate models for the growth of Shigella flexneri in foods.

PROGRESS D: A study of the growth of S. flexneri in sterile foods (meats, milk, meat broths, baby foods) showed that the observed growth kinetics agreed well with those calculated using response surface models for growth at 19 to 37 °C but not at lower temperatures. Additional data for growth in BHI broth at 10 to 19 °C are being evaluated to improve the growth models in the lower temperature range.

OBJECTIVE E: Improve the Listeria monocytogenes growth model.

PROGRESS E: Additional data on the growth of L monocytogenes was collected and fitted to models. Regression equations are being recalculated.
IMPACT/TECH TRANSFER. Objectives B through F are part of a continuing effort to improve our models to give regulatory agencies and industry the ability to predict more accurately the behavior of food-borne pathogens.

Non-Thermal Inactivation Modeling

OBJECTIVE F: Develop an effective model for predicting the inactivation of Listeria monocytogenes when placed in an adverse acidic environment of the type that might be encountered in fermented and non-fermented meat products.

PROGRESS F: Additional data was collected to improve the precision of the predictions. The old and new data sets were combined and new regression equations were calculated.

OBJECTIVE G: To develop a model to predict the Staphylococcus aureus survival during storage of foods not given a thermal process.

PROGRESS G: Survival in BHI broths made with various pH values (3-7), NaCl levels (0.5-20%), lactic acid (0-1%), $NaNO_2$ (0-200 ppm) and stored at 4 to 42 °C was determined. The model fits were recalculated and final regression equations and confidence intervals were calculated. This model was incorporated into Pathogen Modeling Program version 4.0. The results showed rapid inactivations at pH less than or equal to 5. Inactivation rates were rapid at high temperatures and the rate increased with increasing nitrite and total lactate concentrations. Salt had relatively little effect when less than or equal to 8%.

OBJECTIVE H: Develop a survival model for E. coli O157:H7.

PROGRESS H: The previous data base was expanded by adding nitrite and salt as factors and extending the pH range. This will bring fermented meat products into the scope of the model. The final data are being collected. Fitting data to survival models and calculation of regression equations should be accomplished soon. The survival of 15 strains of E. coli O157:H7 in broths simulating salami or pepperoni ranged from 160 to 600 hours (time for 4 log declines). This means that modeling must consider strain differences in order to improve the precision of the predictions. A cooperative project with the National Food Centre, Dublin, Ireland, to determine the survival of E. coli O157:H7 in fermented sausages was initiated with funding from USDA--OICD and EEC. **IMPACT/TECH TRANSFER** for Objectives F, G and H. The capability to predict the survival of pathogens is critical for FSIS and regulatory agencies to evaluate foods with long shelf lives such as fermented meat products. These foods do not permit growth of the pathogens but will contain them from the raw meat or other ingredients. The cooperative with Ireland will answer the critical public health concerns about the potential presence of E. coli 0157:H7 that arose from the outbreak last December in salami.

OBJECTIVE I: Determine the potential for killing bacterial pathogens with pulsed, high voltage electrical fields and how various environmental factors affect the rate of killing.

PROGRESS I: E. coli O157:H7 cells were suspended in various broth media and given a series of electrical field pulses (nominal half-life 15 ms, 2.5 kV/cm). Prior research was expanded by testing higher temperatures and lower pHs. The enhanced rate of inactivation at subpasteurization temperatures (50-60 °C) and at low pHs compared to treatment at ambient temperatures or neutral pHs. **IMPACT/TECH TRANSFER.** This process can pasteurize fluid foods without chemicals, ionizing radiation or normal input, thereby minimizing deterioration in quality.

OBJECTIVE J: To determine the potential for the bacteria, Bdellovibrio, to attack, parasitize and kill gram negative food-borne pathogens.

PROGRESS J: Bdellovibrio are Gram-negative bacteria that prey on other Gram-negative organisms resulting in lysis of prey bacteria. Previous research demonstrated the ability of Bdellovibrio isolated from soil and sewage to attack Salmonella and E. coli O157:H7. Recently marine bdellovibrios were isolated using Vibrio parahaemolyticus as the prey organism. Marine bdellovibrios are useful because of their tolerance to high NaCl levels. Current research includes testing the prey range of the marine bdellovibrios including Salmonella and E. coli O157:H7. Also the use of the bdellovibrios in carcass washes to reduce the number of pathogenic bacteria will be evaluated. The growth of the pathogens will be prevented by adding NaCl to the suspending solution allowing the salt-tolerant bdellovibrios to attack and destroy the prey bacteria. The prey concentration in these washings will be low; however, it was established the Bdellovibrio will attack prey in concentrations of only 100 CFU/ml.

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GROWTH AND TOXIN PRODUCTION OF HARMFUL PSYCHROTROPHS

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OBJECTIVE A: Develop effective barriers and controls of psychrotrophic food-borne pathogens at refrigeration and mild abuse temperatures.

PROGRESS A: Listeria monocytogenes Studies recently completed showed that a long chain sodium polyphosphate (Hexaphos, ave. chain length = 13) exerted a bacteriostatic effect on L. monocytogenes in BHI broth. Growth inhibition increased with decreasing temperature, decreasing pH and increasing sodium chloride concentration. Since sodium polyphosphates are chelating agents, the probable mechanism of inhibition involves the binding of essential metal ions. A study was conducted to assess the effect of added polyvalent metal ions, 1-10 Mm, on growth of L. monocytogenes in BHI media containing 0.5% Hexaphos (max. permitted concentration, nominal 3.6 Mm). Addition of 10 mM Ca^{2+} or Mg^{2+}, 5mM Fe^{3+}, 2 mM Mn^{2+} or 1 mM Zn^{2+} to media of pH 6.0 resulted in growth at 19 °C comparable to that in control cultures without additives. Fe^{2+} (2-10 mM) partly restored growth, while Ni^{2+}, Co^{2+}, Cu^{2+} or Al^{3+} were not effective. While L. monocytogenes did not grow at 28 °C in media of pH 5.0 containing 0.5% Hexaphos, growth occurred when the media were supplemented with Ca^{2+}, Mg^{2+} or Mn^{2+} but not Zn^{2+} or Fe^{3+}. When added to foods (commercially available sterile baby foods were used: beef, sweet potato, butternut squash, green beans, carrots and beets), 0.5% Hexaphos had little, if any, effect on the growth of L. monocytogenes at 19 °C. The concentration of Ca, Mg, Fe and Zn in the foods tested, > 9 mM, was sufficient to reverse the growth inhibition by Hexaphos. However, the concentration of the same metal ions in BHI, 0.43 mM, was low enough for the polyphosphate to inhibit bacterial growth. These observations suggest that polyphosphates, although they perform various useful functions, can not be relied upon to inhibit Listeria in foods.

OBJECTIVE B: Develop bacteriological tests that detect and allow control of temperature abuse of refrigerated foods.

PROGRESS B: E. coli O157:H7 Multiplex PCR Detection Method Studies were continued on the use of a multiplex polymerase chain reaction (PCR) technique for detection and identification of E. coli O157:H7. PCR primers which amplify sequences of a 60-MDa plasmid, Shiga-like toxins (SLT)-I and -II and a gene involved in formation of attaching and effacing lesions (eae) are used in one reaction for simultaneous amplification of the DNA fragments. Experimental conditions for the multiplex PCR were optimized and a multiple digoxigenin-labeled oligonucleotide probe hybridization (DLOPH) assay was developed. E. coli O157:H7 colonies on sorbitol MacConkey agar containing BCIG were identified from experimentally inoculated ground beef and raw milk samples plated following 6-h enrichment culturing. The identities of the PCR products were confirmed using the DLOPH assay. Ground beef and raw milk samples were inoculated with E. coli O157:H7 at different concentrations and were incubated for various time periods. At 0, 2, 4, 6, 8, 12, and 24 h, aliquot of the cultures were removed for DNA extraction followed by testing by the multiplex PCR. E. coli O157:H7 was detected by the PCR after 8 h enrichment culturing when the initial sample inoculum was 1 CFU/g of ground beef and after 6 h of enrichment of raw milk with an initial inoculum of 1 CFU/ml. A DNA amplification method using tableted multiplex PCR reagents was

developed for identification of E. coli O157:H7 isolated from food samples. Use of tableted PCR reagents offers significant advantages over conventional PCR in terms of simplicity and reproducibility of test results. **IMPACT/TECH TRANSFER:** The tablet PCR method was demonstrated to FSIS and is currently under review for use in their regulatory program.

Enterotoxigenic E. coli PCR Method A PCR and an enzyme-linked oligonucleotide probe hybridization assay were developed for the detection of enterotoxigenic E. coli in ground beef, chicken, pork and raw milk. Two synthetic primers, one of which was biotinylated, were used in the PCR to amplify a fragment of the E. coli heat-labile (LT) enterotoxin gene. The identity of the PCR products was confirmed by liquid hybridization using a horseradish peroxidase-linked internal probe in a 96-well microplate coated with streptavidin. The final quantification of the PCR products was performed by a colorimetric reaction. An initial inoculum of as few as 1.2 to 12 CFU of the LT-producing E. coli per 25 g (or ml) of food sample gave a positive reaction.

Bioluminescent E. coli O157:H7 Constructed The firefly (Photinus pyralis) luciferase (luc) gene on a plasmid vector (pBESTluc) was introduced into three strains of E. coli O157:H7, one of which does not possess Shiga-like toxin genes. The plasmid-bearing strains were highly luminescent after application of Beetle Luciferin reagent, indistinguishable from their parent strains in biochemical and serological tests and by the multiplex PCR, and were stable under antibiotic selection. These bioluminescent strains could be useful as positive controls in microbiological assays as well as in studies of bacterial injury and recovery and of the effects of antibacterial agents.

E. coli O157:H7 Carcass Sampling Method A sandwich enzyme-linked immunosorbent assay is being developed to detect the presence of E. coli O157:H7 in beef carcass wash water. The wash water is filtered, the bacteria collected are removed from the filters and the material is tested by the ELISA. Using a chemilumiscent detection system, the detection limit is 10^3 to 10^4 CFU/well.

Biosensor for E. coli O157:H7 Detection A biosensor instrument employing a detection system relying on the optical phenomenon of surface plasmon resonance was used for detection and identification of E. coli O157:H7. The instrument which monitors interactions of ligand with analyte in real time, without the use of labels was used to detect whole bacteria. One of the methods used involves immobilizing antibodies against E. coli O157:H7 onto the sensor chip surface, capturing the bacteria, then using a second O157:H7-specific antibody to enhance the resonance signal. **IMPACT/TECH TRANSFER**: The strains were given to FSIS who is evaluating them for use in their regulatory program.

OBJECTIVE C: Identify biochemical and/or genetic mechanism that allows food-borne pathogens to adapt to psychtrotrophic growth and determine the impact of this adaptation on the organisms~s pathogenicity.

PROGRESS C: Yersinia entercolitica (Ger 0:3) was evaluated at 4 to -12 °C for plasmid loss. Crystal violet and Congo red/low calcium response assay showed that there is plasmid loss over time regardless if the organism is growing, dying, or remains static. The congo red/low calcium response media was verified as highly accurate to predict violence loss, using both autoagglutination and hydrophobicity assays. PCR of selected colonies from the -12 °C experiment confirmed the presence of plasmid (+) cells identified on congo red/low calcium response media. Cells identified as plasmid (-) gave conflicting

results on PCR. This is the first observation of virulence plasmid loss under non-growth conditions.

OBJECTIVE D: Examine culture conditions, especially how low and abuse temperatures affect production of virulence factors and determine ways to control them in psychrotrophic and mesophilic food-borne pathogens.

PROGRESS D: Growth and verotoxin production by hemorrhagic Escherichia coli in ground beef. Four strains of hemorrhagic E. coli were inoculated into commercial ground beef (to yield a starting count of about 10^3/g) and held at 5, 8.5, 12, and 15 °C. Their number were followed by plating at appropriate intervals on sorbitol MacConkey agar (SMac). For some experiments, radiation pasteurized ground beef was used (starting count of background flora reduced to ca 10^3/g) and in certain experiments, strains with an antibiotic resistance marker (obtained by plating on tryptic soy agar + rifampin) were employed (their numbers were followed by plating on SMac + rifampin). Growth was observed at the three higher temperatures, with one strain (O157:NM3) increasing three logs in irradiated ground beef at 8.5 °C within 6 days. There was a small amount of verotoxin produced at the two higher temperatures. **IMPACT/TECH TRANSFER:** These studies indicated that hemorrhagic E. coli can grow in ground beef with low background counts and at mild temperature abuse conditions. Though this bacterium declined gradually in detectable numbers in the presence of the normal background flora of ground beef, low numbers often persisted for several days at all temperatures. Future work will extend these studies to milk as the food substrate.

OBJECTIVE E: Develop a selective enrichment, isolation and identification method for plasmid-bearing virulent strains of Yersinia enterocolitica from ground pork.

PROGRESS E: Recovery of plasmid-bearing strains of Yersinia enterocolitica (YEP+) from ground pork usually requires the use of enrichment technology because of the presence of large numbers of other contaminating microorganisms. Modified Tryticase Soy broth was used for the enrichment of YEP+ from ground pork after the addition of Irgasan at a final concentration of 4 ug/ml, at 7, 24, 48, or 72 hours of incubation at 12 °C. Selectively enriched cultures were diluted and plated at 28 °C on BHA (for total CFU), CIN and MacConkey's agar (for presumptive isolation) at 24 to 72 hours after Irgasan addition. Sorbitol-MacConkey and Mannitol-MacConkey agars were also tested for isolation. Presumptive colonies were streaked onto Congo red agarose medium (CR-BHO) and incubated at 37 °C for 24 hours. YEP+ colonies were identified by a characteristic morphology colony (red pinpoint colonies with occasional white border around the red pinpoint colonies). The virulence of the CR+ colonies were further confirmed by multiplex PCR using chromosomal ail gene (attachment-invasion locus) and virF gene (transcriptional activator for the expression of plasmid-encoded outer membrane protein yop 51) from virulence plasmid. The presence of the ail gene differentiates Y. enterocolitica from Y. pseudotuberculosis and the presence of virF gene detects the presence of virulence plasmid. **IMPACT/TECH TRANSFER:** Thus, the virulent strain is identified by the presence of specific genes and the expression of plasmid-associated virulence property. The enrichment, isolation and identification of YEP+ clones (9 CFU/g) is completed by 6 days whereas, reference method takes 14 days. Thus, the method developed reduces the time by 8 days and describes the specific isolation of YEP+, whereas, the methods reported in the literature are mostly involved for the isolation of Y. enterocolitica which does not imply the isolation of YEP+, since not all Y. enterocolitica are pathogenic. Moreover, the identification of YEP+ on CR-BHO also allows recovery of YEP+ for further investigation, since, the incubation at 37 °C on CR- BHO does not facilitate the loss of plasmid.

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HEAT RESISTANT CLOSTRIDIUM BOTULINUM SPORES IMPACT OF CHANGING TECHNOLOGIES

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OBJECTIVE A: Define the heat treatment required to achieve a specified lethality for non-proteolytic Clostridium botulinum type B and type E spores in turkey to ensure that the heating step is lethal, while avoiding heating that negatively impacts product quality. Use data to develop a mathematical model for determining the effects of environmental parameters on thermal resistance of C. botulinum.

PROGRESS A: Initial data were collected on the heat resistance of a mixture of non-proteolytic C. botulinum type B and type E spores in turkey slurry. Environmental parameters assessed were pH (5.0 - 6.5), sodium chloride (0 - 3%), and sodium pyrophosphate (0 - 0.3%). Survivors were determined using RCM+lysozyme, which had been previously determined to be the most effective recovery medium. The samples were also plated on recovery medium that included the same environmental parameters as the heating menstruum. The latter reflected the heated spores potential to grow out in a specific food (turkey) while the former gives the maximum heat injured spores recovery. Decimal reduction times (D-values) were calculated by fitting a survival model to the data with a curve fitting program. The D-values were analyzed by second order response surface regression equation in temperature, pH, sodium chloride and sodium pyrophosphate levels. The four variables interacted to affect the inactivation of spores. Confidence intervals (95%) predicted heat resistance of spores in turkey. Thermal resistance of spores can be lowered by combining these intrinsic factors. **IMPACT/TECH TRANSFER:** The multiple regression equation developed in this study can predict D-values for any combinations of temperature, salt, sodium pyrophosphate, and pH that are within the range of those tested. Using this predictive model, food processors should be able to design thermal processes for the production of a safe food with extended shelf life without substantially causing adverse effects on the quality of the product.

OBJECTIVE B: Develop a model for the growth of Clostridium perfringens in a media system and assess the effects and interactions of temperature, pH, sodium chloride, and sodium pyrophosphate.

PROGRESS B: Experiments were conducted on modeling growth kinetics of a three strain mixture of C. perfringens in a media system. The effects and interactions of temperature, pH, sodium chloride, sodium pyrophosphate were assessed. Growth data were analyzed by the Gompertz equation; the gompertz B and M parameters were then used to calculate lag phase duration (LPD), exponential growth rate, generation time (GT), and maximum population density values. The data indicated that the growth kinetics of C. perfringens were dependent on the interaction of the four variables, particularly in regard to exponential growth rates and lag phase durations. Cubic models based on the natural logarithm transformation of LPD and GT were generated by SAS, evaluated, and appeared to adequately fit the data. The data suggest that sodium pyrophosphate can have significant bacteriostatic activity against C. perfringens and may provide processed meats with a degree of protection against this microorganism, particularly if employed in conjunction with a combination of acidic pH, high salt concentrations, and adequate refrigeration.

OBJECTIVE C: Influence of modified atmosphere packaging on the growth of Clostridium perfringens in cooked turkey.

PROGRESS C: Clostridium perfringens-containing samples of sterile ground turkey were studied to assess growth under modified atmosphere conditions. Samples were packaged under various atmospheres (CO /O_2 /N_2: 75/5/20, 75/10/15, 75/20/5, 25/20/55, 50/20/30), stored at 4, 15 and 28 °C, and sampled periodically for growth. The growth of C. perfringens was slowest under 75% CO_2 /20% O_2 /5% N_2 at 15 °C. The effectiveness of gas mix decreased with an increase in temperature (28 °C). There was no growth at 4 °C for up to 28 days. Temperature abuse (28 °C storage) of refrigerated products for 8 h did not permit C. perfringens growth. Use of 25 - 75% CO_2 /20% O_2 /balance N_2 may extend the shelf life of turkey, but in the absence of proper refrigeration, it cannot be relied upon to eliminate the risk of C. perfringens food poisoning.

OBJECTIVE D: To determine the influence of cooling rate on growth from spores of Bacillus cereus, Clostridium botulinum and vegetative cells of Staphylococcus aureus, Listeria monocytogenes, and Salmonella serotypes in cooked ground beef.

PROGRESS D: The ability of five food-borne pathogens to grow during cooling of cooked beef was studied to determine a safe cooling rate. Beef samples were inoculated with heat-shocked spores of B. cereus (strain BH 86) or C. botulinum (non-proteolytic type B strains CBW 25, 17B and KAP B5; type E strains Whitefish, Saratoga and Alaska), or vegetative cells of L. monocytogenes (strains HO-VJ-S, V-7 and Scott A), S. aureus (strains 196E, B121and B124) or Salmonella serotypes (S. dublin, S. enteritidis and S. typhimurium), vacuum-packaged, and cooked in a circulating water bath to an internal temperature of 60 °C in 1 h. Heated samples were cooled through the temperature range of 54.4 °C to 7.2 °C at rates varying from 6 to 18 h. Samples were removed at various times during cooling to determine if growth of the pathogens had occurred. No growth was observed with cooling periods of up to 21. **IMPACT/TECH TRANSFER:** Pasteurized cooked beef can be cooled to 7.2 °C in up to 21 h without a food safety hazard due to growth of these pathogens. Our study simulated the cooked roast beef environment in the food industry and provided guidelines for safe cooling rates of roast beef.

OBJECTIVE E: Determine the growth potential of C. perfringens from a spore inoculum in sous-vide turkey products at refrigerated and cyclic and static temperature abuse storage conditions; also, define the heat resistance of C. perfringens spores in turkey containing 0 - 3% salt (sodium chloride) levels.

PROGRESS E : Clostridium perfringens growth from a spore inoculum was investigated in vacuum-packaged, cook-in-bag ground turkey (pH 6) that included 0.3% (w/w) sodium pyrophosphate, and sodium chloride at 0,1, 2 or 3% (w/w). The packages were processed to an internal temperature of 71.1 °C, ice chilled and stored at various temperatures. At 28 °C, the addition of 3% salt in turkey was effective in delaying growth for 12 h. At 15 °C, growth occurred at a relatively slow rate in the presence of 1 - 2% salt. Vegetative cells were not observed even after 28 days of storage in the presence 3% salt. C. perfringens growth was not observed at 4 °C regardless of salt levels. The D-values ranged from 23.2 min (no salt) to 17.7 min (3% salt). Cyclic and static temperature abuse of refrigerated products for 8 h did not lead to growth by C. perfringens from a spore inoculum.

OBJECTIVE F: Develop rapid techniques to detect and specifically identify strains of Clostridium perfringens type A.

PROGRESS F: A gene amplification procedure based on Polymerase Chain Reaction (PCR) technology has been developed for specific identification of the enterotoxin A gene of C. perfringens. A 750 base pair fragment internal to the enterotoxin A gene is amplified by PCR following a short enrichment incubation combined with a chromosomal DNA extraction step. The PCR amplification is performed in two hours and is followed by detection of the amplified DNA. An enzyme linked immunoassay (PCR-ELISA) utilizing a chemiluminescent substrate has been developed for detection and confirmation of the PCR-amplified DNA. Confirmation of the PCR-amplified DNA relies on its hybridization to an internal probe that will generate the chemiluminescent signal upon addition of the substrate. This confirmation assay is performed within three hours after the PCR amplification procedure. The PCR-ELISA was demonstrated to be as sensitive as conventional Southern hybridization while providing faster results. The PCR-ELISA demonstrated a level of sensitivity in the range of r10 CFU/g of meat in the presence of the background flora (~10^6 CFU/g) present in raw beef. **IMPACT/ TECH TRANSFER:** Method was demonstrated to FSIS, who is evaluating it for use in the regulatory program.

OBJECTIVE G: Assess the reliability of two DNA-based detection methods, PCR versus colony hybridization, for assessment of viability of C. perfringens in irradiated beef samples.

PROGRESS G: A nonradioactive colony hybridization and a PCR amplification procedure were compared against conventional plate count for assessment of viability of C. perfringens in gamma irradiated beef samples. The research established a correlation with viability when detection of a food-borne pathogen is applied to previously irradiated food. The colony hybridization and plate count method, by detecting growth of viable cells, provided a direct correlation with survival of enterotoxigenic C. perfringens. Detection by PCR did not show a reliable correlation with viable plate counts or the colony hybridization assay. Increases in the size of the amplified PCR fragment (183 bp versus 750 bp) showed correlation with DNA damage due to exposure to radiation as shown by amplification signals that were decreased and in some cases absent. However, meat samples containing high levels of microbial contamination showed positive PCR amplification signals after exposure to levels of radiation where no viable cells were recovered by the plate count or colony hybridization methods.

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CONTROL OF PATHOGENS AND MAXIMIZATION OF VITAMIN RETENTION IN IRRADIATED MEAT AND POULTRY

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OBJECTIVE A: To determine the loss of alpha-tocopherol and thiamin in exotic meats due to gamma irradiation.

PROGRESS A: We investigated the loss of alpha-tocopherol and thiamin in bison, alligator, caiman and ostrich to determine if there are any differences between these exotic meat animals and conventional domestic species such as beef, pork and chicken, either in vitamin content or the loss thereof during gamma irradiation. We also determined the proximate composition. The proximate compositions were similar to those of domestic species, except that the exotic meats had lower fat contents. The loss of thiamin and alpha-tocopherol were the same for the exotic species as for the domestic species.

IMPACT/TECH TRANSFER: The study supports the concept of "chemiclearance", that the effects of radiation appear to be comparable for all meats for pasteurization purposes. It is background information for Federal agencies in issuing regulations for the use of ionizing radiation.

OBJECTIVE B: To determine the effect of temperature on alpha-tocopherol and thiamin loss in frozen pork due to gamma irradiation.

PROGRESS B: We investigated the loss of thiamin and alpha-tocopherol in frozen pork in the temperature range from -20 to -60 °C. The loss of thiamin showed a low temperature dependence, with a heat of activation of 2200 calories per mole, typical of radical reactions, indicating that the reaction was the oxidation of thiamin by the hydroxyl radical produced from the gamma irradiation of water. The loss of alpha-tocopherol showed a much lower temperature dependence, with a heat of activation of 600 calories per mole or less. The loss of alpha-tocopherol leveled off after ca. 2-3 kiloGray due to depletion of residual oxygen in the tissues. The data show that temperature has little or no effect on the loss of either vitamin in the frozen state.

IMPACT/TECH TRANSFER: The information will be useful for establishing parameters for irradiating frozen meats.

OBJECTIVE C: Develop a method for the analysis of cholesterol and its oxidation products.

PROGRESS C: Gamma irradiation of cholesterol has been shown to result in the formation of cholesterol oxides. The separation of cholesterol and ten of its oxidation products ranging from weakly to moderately polar was achieved by normal phase gradient high performance liquid chromatography with an evaporative light scattering detector. This universal mass detector does not detect changes in solvent composition, making it possible to employ solvent gradients, essential whenever a wide range of compounds with diverse characteristics are to be separated. Standards ranging in concentration from 0.1 to 1.0 microgram were chromatographed on an alumina/silica column using a gradient containing dichloromethane, acetonitrile and water. Complete separation was obtained

within 37 minutes.

IMPACT/TECH TRANSFER: The method makes possible the isolation of a wide range of cholesterol oxides. This is of interest to biochemists and clinicians because of the role in health of cholesterol oxides produced as a part of normal body oxidative processes.

OBJECTIVE D: To develop a practical method to identify irradiated poultry.

PROGRESS D: A method was previously developed in our laboratory to measure carbonyl levels in poultry breast tissue as a test to determine if poultry had been subjected to ionizing radiation. We are currently conducting "blind" studies to evaluate the degree of accuracy of this test to differentiate between irradiated and unirradiated samples. Intact breasts, from freshly slaughtered chickens, are being irradiated (Cs-137) at 0, 1.5, and 2.5 Kgy at 5 °C. The chickens are stored at 4 °C for 3-7 days and then assayed to determine the efficacy of this test.

IMPACT/TECH TRANSFER: The method when developed can be used to determine if chickens had been processed with ionizing radiation.

OBJECTIVE E: To determine the radiation resistance of the food spoilage bacterium Shewanella putrefaciens.

PROGRESS E: Shewanella putrefaciens is a common spoilage bacterium associated with meats. As its name implies it produces noxious odors. The resistance of S. putrefaciens ATCC 8071, 8072, and 8073 to gamma radiation was determined in the presence and absence of air on mechanically deboned chicken meat (MDCM). The presence or absence of air (oxygen) did not significantly influence resistance to gamma radiation at 5 °C, and it was very sensitive with a D_{10} value of 0.114 plus/minus 0.002 kGy on MDCM. A high percentage of cells surviving irradiation were shown by impedance measurements to have suffered injury. The bacteria were significantly more resistant to gamma radiation at temperatures below the freezing point. At a dose of 0.8 kGy lowering the temperature of irradiation by 10 degrees increased the survival of this organism by 1.66 $\log_{10}$. The type of meat (hamburger, ground beef round, ground pork, and ground turkey breast) did not significantly alter resistance of S. putrefaciens to gamma radiation under identical conditions (D_{10} value = 0.0178 plus/minus 0.007 kGy).

IMPACT/TECH TRANSFER The minimum radiation dose currently approved for poultry in the USA, 1.5 kGy, should eliminate S. putrefaciens from meats.

OBJECTIVE F: To develop a method of estimating the extent of bacterial injury from radiation or heat using impedance measurements.

PROGRESS F: A method was developed for the estimation of bacterial injury based on impedance measurements using modern automated systems. The system avoids the labor intensive systems based on estimations of colony forming units (CFU) on both non-inhibitory and inhibitory media. Estimates can be made using standard curves for non-injured cells to predict from observed detection times the expected versus the actual observed CFU numbers. The difference between these values represents the injured population.

IMPACT/TECH TRANSFER This technique will benefit scientists studying injury of foodborne pathogens and ultimately the consumer by providing a safer food supply.

OBJECTIVE G: To identify the mechanisms by which irradiated salmonellae become more sensitive to heat than are non-irradiated cells.

PROGRESS G: Effects of radiation and heat on survival of Salmonella typhimurium ATCC 14028 were examined by measuring DNA damage in vivo and the integrity of the cytoplasmic membrane. Radiation sensitivity of S. typhimurium was greater in the presence of oxygen than in the presence of nitrogen gas (D_{10} equals 0.394 plus/minus 0.029 in air and D_{10} equals 0.561 plus/minus 0.035 in nitrogen). To examine DNA damage in vivo, plasmid pBR322 was transformed into S. typhimurium by electroporation. Recovery of the covalently closed circular (ccc) form of plasmid pB322 from S. typhimurium transformants (Amp r, Tet r) was decreased by irradiation, but not by heating. Heating prior to irradiation significantly decreased the recovery of plasmid DNA without affecting survival of S. typhimurium. Transformability of the recovered plasmid pBR322 was affected by neither irradiation nor heating and mutation of antibiotic resistance genes was not detected in S. typhimurium. Heating, but not irradiation, caused destabilization of the cytoplasmic membrane. These results suggest that lethality of heating followed by irradiation for S. typhimurium was additive reflecting, radiation-induced DNA damage and heat-induced membrane destabilization. When irradiation preceded heating in the absence of oxygen, more cells were inactivated, the radiation damaged DNA was more sensitive to heat than would be expected from the sum of the two processes.

IMPACT/TECH TRANSFER A better understanding of the processes by which bacteria are killed may lead to more effective food processing technologies benefiting the meat and poultry processing industries.

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SURFACE PASTEURIZATION

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OBJECTIVE A: To develop a rapid and economic method for reducing the level of microbial contamination on the surface of poultry meat without introducing significant degradation of the quality of the product.

PROGRESS A: A pasteurization system has been conceived, publicly patented, built, and tested to determine the feasibility of treating raw poultry meat with steam so briefly that surface organisms are killed; but no appreciable cooking of the meat occurs. Four log reduction of applied Listeria inocuans was achieved on fresh poultry meat without cooking by application of 138 °C steam for 26 milliseconds. Somewhat poorer kills were found on fresh beef and pork, although conditions for these meats have not yet been optimized. It was found that low temperature steam should be used to flush out traces of air remaining around the meat after initial evacuation by vacuum for one second. Treatment steam must be thermally saturated for heating rapid enough to avoid cooking. One second vacuum was used in the cooling step. A new pasteurization system to treat whole eviscerated chickens has been designed. Construction of this system, which will serve as a prototype for a commercial operation, will begin soon.

IMPACT/TECH TRANSFER: Confidential discussions are underway with potential equipment manufacturers.

PUBLICATIONS:

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Morgan, A.I. Canadian patent application, 2,116,838, Method and apparatus for treating and packaging raw meat.

DETECTION OF HUMAN VIRAL PATHOGENS IN MEAT

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CRIS Number: 363042000-001
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OBJECTIVE A: Develop oligonucleotide primers for use in nucleic acid amplification tests that will detect and differentiate Norwalk and Norwalk-like viruses in meat products.

PROGRESS A: The major foodborne human viral pathogens, Norwalk virus, and the other small, round structured viruses (SRSV) belong to the calicivirus family. These viruses cause severe gastroenteritis characterized by vomiting and diarrhea. The caliciviruses are drawing increased attention because recent findings indicate they are involved in more disease than previously known and because of the discovery that these viruses have wider host ranges than previously believed. An example of this is the finding that RNA polymerase sequences from two Japanese SRSV are more similar to the animal caliciviruses than to other human SRSV. This finding is of concern because it indicates there are potential sources of infection for humans that were unknown 2 to 3 years ago.

The SRSV are difficult to work with because they cannot be propagated in vitro. The only source of virus is from samples from stools obtained from disease outbreaks, or from stool specimens obtained from human volunteers after controlled infections. To develop specific detection procedures, a model system is needed which will provide a constant supply of virus. The porcine and bovine enteric caliciviruses (PECV and BECV, respectively), are caliciviruses which may serve asviral models. Also, there is some concern that PECV and BECV could be spread in meat products and cause disease in humans. Work with PECV has started. This should lead to determination of relatedness to the human viruses and allow design of PCR primers for ready detection.

For work involving BECV, samples of bovine stool have been obtained that contain caliciviruses by electron microscopy. One of these samples has been inoculated into calves from which samples were collected for analysis. The fecal samples will be used as a source of virus for molecular cloning and nucleotide sequence analysis to develop PCR primer sets and other diagnostic reagents.

Work was undertaken to further define the relatedness of the human viruses to animal caliciviruses. A PCR primer set that was developed amplified product from 17 animal caliciviruses examined. A second PCR primer set amplified product from 16 of 17 animal caliciviruses examined. The design of these primer sets represents significant progress in diagnosis and differentiation of caliciviruses, because until now the method of diagnosis of caliciviruses was negative staining and electron microscopy. That procedure does not allow differentiation of animal pathogens from human pathogens. The PCR primers will find utility by agencies or laboratories in the diagnosis and differentiation of possible calicivirus infections. The amplification products described above were cloned, sequenced, and compared with each other and with the Norwalk-like family of viruses to determine extent of relatedness. Phylogenetic analysis was done with two regions of the viral genomes. The animal caliciviruses were found to be very closely related to each other; however, similarity of the animal caliciviruses with the Norwalk family of viruses was limited. results demonstrate animal caliciviruses have some value as models for the

human caliciviruses at the genetic level but are unlikely to have value at the antigenic level. Agencies or laboratories using PCR to diagnose calicivirus infections can now be assured that known animal caliciviruses will not cause false positive results in PCR-based tests developed for the human caliciviruses. Further analysis of the genetic relatedness of newly discovered animal caliciviruses to human caliciviruses is an ongoing effort.

IMPACT/TECH TRANSFER: Information obtained from this research adds to the knowledge of the relatedness of caliciviruses and whether the animal caliciviruses pose a threat to human health. Also of value are the efforts to develop a model system for the study of these viruses. A model system is desired to expedite the development of new diagnostic protocols and reagents. The primer sets described above for animal calicivirus have been transferred to APHIS, FAADL, DVL, Greenport, NY, for further development and testing. Calicivirus plasmid constructs and antisera were supplied to scientists at NIH, NIAID, Bethesda, MD.

OBJECTIVE B: Develop sensitive and specific immunological reagents for the rapid detection of viral foodborne pathogens in meat products.

PROGRESS B: Probably the greatest need in screening food products for pathogens is ability to test a large number of samples in as short a period of time as possible. An important aspect of rapid detection methods is to assure that sensitivity of the test is sufficient to detect low numbers of pathogens. We have chosen to concentrate our efforts on development of rapid detection methods for the SRSVs. There are two reasons for this. First, molecular reagents are now available for SRSVs which will allow us to develop rapid and sensitive detection protocols without working directly with virulent viruses. Availability of these molecular reagents is timely because the SRSVs, as mentioned above, cannot be propagated in cell culture and are obtained only from infected human volunteers. These molecular reagents make it possible to develop detection tests without extensive and costly renovations to laboratories, which would be necessary with work involving the live, virulent viral agents.

The second reason for concentration on SRSVs is that related animal caliciviruses are available which can be grown in cell culture, allowing development of model systems for detection tests for the SRSVs. Work is currently underway on the development of immunological reagents (antibodies) and reporter systems to rapidly and accurately detect pathogens in foods. These reagents utilize bioluminescent molecules (luciferase, aequorin, and green fluorescent protein) as the reporters. The reporter molecules will be linked to the immunological reagents, which will provide the specificity for the tests. These immunological reagents are in the form of polyclonal antisera against viral proteins or monoclonal antibodies against specific antigens (sites on the viruses).

Currently under development are monoclonal antibodies produced against the capsid protein of Norwalk virus. Another reagent, termed single chain variable fragments (ScFv), also will be developed. This involves the cloning and expression of the variable regions of antibody genes. These ScFv will be constructed in a way that when expressed, the protein will still recognize and bind antigen specifically. The genes for these constructs can be obtained from the hybridoma cells that secrete specific monoclonal antibodies used above. This ScFv gene can then be fused to any of the genes encoding the bioluminescent proteins listed above. The expressed fusion protein binds antigen specifically and can generate light under proper conditions. We are also investigating a reagent that consists of a protein A:luciferase fusion protein. This reagent, which is in hand, binds IgG specifically and produces light when mixed with ATP and luciferin. Preliminary results indicate that rabbit IgG bound to latex beads was detected at the low

level of approximately 25 pg of protein. Constructs consisting of fusion between Protein A and the bioluminescent molecules green fluorescent protein and aequorin have been made and are being tested.

IMPACT/TECH TRANSFER: The work detailed above represents new technologies which will be employed in new extremely sensitive tests which can be used to screen large number of samples, be conducted in a short period of time, and will not require expensive equipment and extensive background knowledge to conduct.

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PASTEURIZATION CONDITIONS FOR KILLING PARATUBERCULOSIS IN MILK

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FSIS Number: NA
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OBJECTIVE A: Determine optimal pasteurization conditions for killing M. paratuberculosis in milk.

PROGRESS A: Experiments to determine optimal pasteurization conditions for milk containing M. paratuberculosis have been conducted. In vitro experiments have shown that large inoculums of M. paratuberculosis (10^8/ml) will survive current pasteurization standards of 71.7C, 15 seconds, but are adequately killed by old industry standards of 65 °C, 30 minutes. Isolates of M. paratuberculosis from a Crohn's patient (strain Ben) are more susceptible to heat treatment than the laboratory strain tested (strain 19698). Field strains of M. paratuberculosis isolated from clinically infected animals are also being tested. Experiments with a small-scale pasteurizer unit are being initiated.

PUBLICATIONS:

Stabel JR. 1995. Crohn's disease and paratuberculosis: The debate continues. Proceedings of American Veterinary Medical Association, July 1995, Pittsburgh, PA. (In Press).

ADVANCED TECHNOLOGIES FOR RESIDUE DETECTION

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OBJECTIVE A: Develop SFE instrumentation and components suitable for use in regulatory laboratories.

PROGRESS A: The commercial supercritical fluid extractor (SFE) developed jointly between ARS and Applied Separations, Inc. employs a pumping system that requires the use of carbon dioxide cylinders pressurized with a helium headspace as do most of these instruments sold in the marketplace. Lately, problems have been reported with the use of helium pressurized cylinders. Among them are: less efficient pump operation, reduced solubility of certain analytes due to the presence of helium in the fluid stream and the additional cost of adding helium to cylinders, particularly in overseas markets. To overcome these problems, ARS and the company have jointly developed a chiller system assembly for the pump module of the Applied Separation's SFE enabling the instrument to operate without helium charged cylinders. The chiller assembly is now in commercial production.

IMPACT/TECH TRANSFER: Two chiller assemblies were fabricated in ARS facilities. The assemblies will be sent to the FSIS Eastern and Midwestern laboratories in order to retrofit earlier models of the Applied Separations SFE purchased by those facilities. Three scientists from the FSIS Eastern laboratory, Athens, GA visited ERRC for one week of intensive training in the use of the Applied Separations SFE for extracting trace level residues from animal tissues. This training was undertaken so that the scientists could begin a program at their facility to evaluate residue isolation using SFE. An ERRC scientist visited the FSIS Microbiology Division laboratory, BARC-East, Beltsville, MD on three occasions to discuss research on methods to detect unidentified microbial inhibitors (UMI).

OBJECTIVE B: Apply SFE for the isolation of sulfonamide drug residues in animal tissues.

PROGRESS B: In previous studies, three sulfonamides; sulfamethazine, sulfadimethoxine and sulfaquinoxaline were extracted from fortified chicken liver with high recoveries using supercritical carbon dioxide. The sulfonamides were trapped on an in-line sorbent bed, eluted with mobile phase after SFE and directly injected onto an HPLC. During recent studies, attempts were made to extract the sulfonamides from beef and pork liver using the experimental conditions employed with chicken liver. Recoveries of these compounds were poor compared to recoveries from chicken liver indicating that the drugs were bound to the matrix differently in the three tissues. Subsequent investigations with different experimental parameters increased recoveries; however, they did not equal those obtained from chicken liver.

OBJECTIVE C: Develop an SFE method for the isolation of melengestrol acetate in bovine fat tissue at the tolerance level.

PROGRESS C: Studies on the determination of melengestrol acetate residues by SFE from fortified and incurred bovine fat tissues have been completed. The procedure employs a combination of supercritical fluid extraction (SFE) and solid phase extraction (SPE) techniques to produce an extract suitable for either HPLC-UV or GC-MS analysis. Overall recovery of the analyte from bovine fat tissue at 100 ppb or lower was 99.4% with a coefficient of variation of 4.14%. The SFE-SPE procedure utilizes a total of 12 ml of organic solvent per fat tissue sample versus greater than 1.9 liters consumed in the current FSIS extraction procedure. **IMPACT/TECH TRANSFER:** The method is being transferred to the FSIS Midwestern Laboratory for evaluation and eventual implementation.

OBJECTIVE D: Develop an assay for spectinomycin in chicken tissues at the regulatory level using electrochemical detection.

PROGRESS D: A method has been developed employing liquid/liquid extraction and electrochemical detection for the analysis of spectinomycin in chicken muscle at a detection limit of 1.0 ppm. Reproducibility of the method is poor due to detector instability. To date, it has not been possible to determine whether the detection problems are due to this specific detector or to the technology itself. This question may be answered in future trials using electrochemical detectors having features not available on the detector currently in use.

OBJECTIVE E: Evaluate non-immunological affinity methods for the multiresidue isolation of beta-lactam antibiotics from the fluids and tissues of food-producing animals.

PROGRESS E: Non-immunological bioaffinity techniques were investigated for the isolation of trace levels of antibiotics from the fluids and tissues of food-producing animals. The studies were aimed toward determining the factors participating in the binding interaction between a solid support-immobilized protein and the antibiotic. Clear differences in the drug-binding behaviors of immobilized and soluble proteins were observed and factors influencing the immobilized protein's ability to bind the drug were established. Different immobilization methods were investigated and utilization of the immobilized protein for the isolation of penicillins from spiked swine serum was evaluated.

IMPACT/TECH TRANSFER: This work has established guidelines for evaluating or developing non-immunoaffinity procedures for drugs and have provided substantial insight in the biorecognition properties between immobilized proteins and trace amounts of low molecular weight analytes.

OBJECTIVE F: Investigate the use of automated on-line microdialysis for analyte sample preparation (analyte extraction and concentration) prior to HPLC detection.

PROGRESS F: Preliminary work evaluating sample preparation using microdialysis (ASTED, Gilson, Inc.) is in progress. Optimum dialysis and detection conditions using the system were determined for standard solutions of the antibiotics benzylpenicillin and flumequine. Processing of swine serum spiked with 1 and 0.1 ppm flumequine by the ASTED system resulted in 50% and 35% recoveries of the drug, respectively, when compared to buffered standard solutions of flumequine dialyzed in an identical manner. A simple protocol was followed based on alkaline extraction and no use of organic solvents. The recovered flumequine at the 0.1 ppm spiking level was clearly resolved on a polymeric HPLC column and easily detected by either UV absorbance or fluorescence.

IMPACT/TECH TRANSFER: The use of automated on-line microdialysis has the potential for the solvent-free trace chemical residue analysis in foods for regulatory applications.

OBJECTIVE G: Develop method for the analysis and classification of beta-lactam antibiotics in samples determined by the 7-plate assay to contain (unidentified microbial inhibitors) UMI.

PROGRESS G: An enzyme digestion technique was developed to classify cephalosporins in samples positive for beta-lactams and "UMI"s followed by an integrated bioassay and HPLC method for the identification of beta-lactams confirmed by penicillinase and cephalosporase enzyme digestion. Thirty incurred samples (including 7 UMI positive) have been analyzed; 2 UMI- containing samples were positive for cephalosporins and determined by HPLC to be ceftiofur metabolite and cloxacillin.

IMPACT/TECH TRANSFER: This approach is proposed for regulatory use for the identification of most beta-lactams. A formal agreement has been developed with the FSIS Microbiology Division for transfer of the enzyme modification method to the field laboratories.

OBJECTIVE H: Develop methods for the analysis and classification of aminoglycoside antibiotics found positive by the 7-plate assay.

PROGRESS H: A polyclonal sheep antisera against hygromycin was characterized and shown to be highly specific for hygromycin B with no cross reaction with other aminoglycosides. Optimum conditions for a competitive immuno-assay using the BIAcore biosensor were determined for detection of hygromycin B. The detection capacity was from 2.5ng/ml to 5 ug/ml. This assay will be applied to tissue samples. Polyclonal sheep antisera against spectinomycin are being produced for use in immunoaffinity purification. The antisera are being characterized at ERRC using the BIAcore instrument.

IMPACT/TECH TRANSFER: The hygromycin B antisera is being evaluated by Diagnostic Specialties and International Diagnostics for commercialization of an ELISA kit for hygromycin B. Diagnostic Specialties is also preparing immunoaffinity silica columns for ERRC (at no cost) for use as an aqueous clean- up of hygromycin B in biological samples.

OBJECTIVE I: Detection of pathogenic bacteria in meat using immunoelectrochemical sensors.

PROGRESS I: Immunoelectrochemical sensors have been developed for rapid detection of Salmonella and E. coli O157:H7. These consist of graphite disk electrodes coated with antibody against a wide range of Salmonella serovars. An assay involves exposing the electrode to four solutions, with washing between each. These solutions are: sample solution (target organism is captured by the antibody); blocking solution (prevents non-specific binding of conjugate); enzyme-antibody conjugate solution (specifically attaches an enzyme label to the cells); and substrate solution (a non-electroactive substrate that is converted to an electroactive product by the enzyme). The product formed near the electrode surface is detected electrochemically within minutes of substrate addition. In addition to development of the sensors, a wide variety of supporting methods and apparatus were developed.

An ELISA test was developed to characterize the antibodies and conjugates used, and to establish starting values for concentrations, incubation time, pH, etc. Methods for rapidly and non-destructively staining and imaging bacteria on the sensor surface by epifluorescence microscopy were developed, and proved very important in studies of capture efficiency.

Rapid scan electrochemical techniques were developed for characterizing the electrochemical performance of the sensor and for optimizing detection sensitivity. By using square wave voltammetry, the initial sensitivity of the method was improved 50 fold. The detection limit for both Salmonella and E. coli O157:H7 was approximately 100,0005 cells/mL and the assay required two hours to complete. The electrochemical detection step was capable of sensing fewer than 100 captured cells, but capture efficiency was less than 1%, and this factor limited the sensitivity of the assay. A filtration capture system was recently investigated, and initial results were promising. Sample solutions were passed through 0.2 mm membrane filters, capturing virtually 100% of the bacteria. After addition of enzyme-antibody conjugate solution to the filter followed by washing, the filter was placed against a graphite electrode, and the assembly immersed in substrate solution. Electrochemical measurement of the enzyme product was performed as above. Based on results for samples containing 100,000 cells/mL, detection limits of 1000 cells/mL or lower are anticipated for this approach. **IMPACT/TECH TRANSFER:** This test approach has the potential to detect low levels of specific bacteria in processing environments.

OBJECTIVE J: To develop assays for bacteria based on antibody-mediated capture with magnetic particles followed by electrochemical detection.

PROGRESS J: Antibody-coated magnetic particles have proven very useful in isolating and concentrating bacteria prior to culturing or PCR analysis. The particles can be dispersed in a large (1-10 mL) sample volume, allowed to bind the target bacteria, and then concentrated into a volume of a few mL by applying a magnetic field. This technology has been coupled with enzyme-antibody conjugate labeling and immunoelectrochemical detection to produce a rapid assay for Salmonella. By placing a magnet behind the electrode, bacteria bound to magnetic particles are pulled close the

electrode surface where they can be efficiently detected. An apparatus was designed and constructed to process 10 samples at a time. A number of electrode types were evaluated, and disposable electrodes consisting of screen-printed graphite on flexible plastic sheets were found to give the best combination of electrochemical performance and magnetic permeability. The capture and detection process was optimized using enzyme-labeled particles as test analytes and employing the same antibody conjugate and electrochemical detection methods used in the immunoelectrochemical sensor. It was determined that background signal from non-specific binding of conjugate to the particles was limiting performance. Extensive testing of a wide variety of blocking agents was therefore pursued, and casein was found to be the most effective material for preventing non-specific binding to the particles. Using commercially available anti-Salmonella coated magnetic particles, a detection limit of 3600 S. typhimurium was achieved. Analysis of one sample required 80 minutes, while ten samples could be processed in 100 minutes.

IMPACT/TECH TRANSFER: A confidentiality agreement has been signed with IGEN Inc., a manufacturer of analytical instruments utilizing magnetic particle capture and electrochemiluminescence detection. ARS and IGEN scientists will evaluate IGEN's technology and determine whether a CRADA is appropriate.

OBJECTIVE K: To develop sensors for bacteria based on antibody-mediated capture and detection at a piezoelectric crystal surface.

PROGRESS K: The oscillation frequency of piezoelectric crystals is highly sensitive to crystal mass, and this effect can be used to detect binding of cells to the surface of an antibody-coated crystal. Conventional piezoelectric sensors apply a sinusoidal voltage to gold electrodes on the crystal surface and measure the resulting oscillation frequency. We have built and tested a novel circuit which simultaneously controls the voltage at the gold electrode, measures the current, and records the oscillation frequency. This arrangement has a number of advantages over conventional systems. First, the ability to make combined electrochemical and frequency measurements can significantly improve the selectivity of detection, since two independent signals are generated. Second, this circuitry permits the potential of the crystal surface to be precisely controlled, so that effects such as electrostatic repulsion of cells can be minimized. Third, by setting the potential of the electrode appropriately, it may be possible to actively transport cells to the surface by electrophoresis, enhancing capture efficiency significantly. Protocols for covalent attachment of antibodies to crystal surfaces have been developed, and capture of cells at these surfaces studied by epifluorescence microscopy and immunochemical techniques. Novel antibodies to irradiated E. coli O157:H7 and Salmonella have been prepared and partially characterized. These antibodies are expected to have improved response to living cells in comparison to commercial antibodies prepared against heat-killed organisms. **IMPACT/TECH TRANSFER:** This method has the potential to detect low levels of bacteria in foods.

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DEVELOP IMMUNOCHEMICAL-BASED RESIDUE METHODS FOR ANALYSIS OF VETERINARY DRUG AND PESTICIDE RESIDUES IN FOOD ANIMALS

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OBJECTIVE A: To develop, evaluate, and provide confirmatory testing of monoclonal antibody-based immunoassays useful for either on-the-farm or in-the-processing-plant, and for laboratory-based analysis of pesticide/drug residues in animal products and body fluids.

PROGRESS A: Development of monoclonal antibody-based immunoassays are in progress for the following compounds: halofuginone, hygromycin B, pirlimycin, fumonisin, furosemide, ceftiofur, salinomycin, monensin, sulfa drugs identified by FSIS as being important for development of immunoassays. The immunoassays for halofuginone, ceftiofur, and salinomycin are the most complete.

Halofuginone: The halofuginone immunoassay is formatted as an ELISA with sensitivities in the 1 to 10 ppb range. An HPLC method for analysis of chicken serum has been completed and published. Using this method analyses of fortified chicken serum samples have been completed with 90-100% recoveries. Results indicate that the drug is rapidly cleared from the serum. Therefore, serum would not be good to monitor for halofuginone residues and studies are underway to develop a simplified sample extraction method for halofuginone analysis in chicken liver tissue with our immunoassay. To this end, incurred chicken liver samples have been generated and are currently being evaluated using the ELISA. These results will then be compared with the values obtained using an HPLC method. **IMPACT/TECH TRANSFER:** Results from these studies have been published and we are actively seeking kit producers for this product.

Salinomycin: The salinomycin immunoassay we developed also is formatted as an ELISA. Sensitivities are in the low ppb range. This is a simple, rapid assay that is able to detect salinomycin in chicken liver. In its present format the assay does not require any complex sample preparation or use of organic solvents. Liver samples are simply homogenized in buffer, diluted, and assayed. Studies using fortified liver samples resulted in recoveries of 80-100%. Incurred liver samples have been tested using both our ELISA method and an HPLC method also developed in our laboratory. Good correlations between the two methods were obtained. However, the ELISA was far more sensitive that was the HPLC. **IMPACT/TECH TRANSFER:** The Salinomycin studies have been recently published. An application for a US patent covering the salinomycin ELISA has been made and has been allowed by the patent office. This antibody has been exclusively licensed to Neogen Inc, and they are currently marketing a kit for detection of salinomycin.

Ceftiofur: Ceftiofur is an FDA approved veterinary cephalosporin antibiotic for the

treatment of respiratory diseases in cattle, horses, and swine. We have developed a monoclonal antibody utilizing as immunogen a protein conjugate of the ceftiofur metabolite desfuroylceftiofur. The monoclonal antibody has been formatted into an ELISA assay. Our preliminary experiments with raw milk indicate that ceftiofur can be detected in milk samples without the need for any extraction or cleanup steps. We are currently evaluating the performance of this ELISA using raw milk samples from individual cows containing incurred ceftiofur residues. These same incurred residue milk samples also were sent to other ARS laboratories for use in development of an HPLC method for ceftiofur in milk. We also have initiated studies to detect ceftiofur in a variety of beef tissue samples. **IMPACT/TECH TRANSFER:** This antibody was developed under a CRADA and it is currently being formatted into a commercial immunoassaay kit by our CRADA partner. We have been authorized to seek patent protection for these antibodies.

Furosemide: We previously developed a monoclonal antibody to furosemide (lasix) and have completed a study demonstrating that an ELISA based on this antibody is capable of detecting lasix in raw milk. Good correlations between the ELISA method and an HPLC method we developed were observed. **IMPACT/TECH TRANSFER:** This data has been submitted for publication.

Other Compounds: Monoclonal antibodies have been developed for monensin. They are currently being evaluated by one of our CRADA partners. We have developed monoclonal antibodies and an ELISA for fumonisin. The assay detects fumonisin B1, B2 and B3. We have completed synthesis of an immunogen suitable for generation of monoclonal antibodies to pirlimycin. Using this conjugate, we have immunized a number of mice and anticipate antibody production and assay development in the coming year. We have generated a new immunogen in our effort to produce monoclonal antibodies to the sulfa drugs, e.g., sulfamethazine. The immunoassay for the sulfa drugs is being developed in collaboration with a CRADA partner. This conjugate has been synthesized de novo and in our laboratory and it was anticipated that it should have produced antibodies capable of recognizing most members of this important class of animal drugs. However, experiments completed to date indicate that no such pan-specific antibody was produced. We have obtained new immunogens from our CRADA partner. These should allow us to produce antibodies specific to individual sulfa drugs. We have immunized mice with three such immunogens and are now initiating experiments to produce new hybridomas. Our CRADA partner has indicated that a collection of antibodies, each specific for a given sulfa drug would be useful and would be incorporated into a multianalyte test capable of detecting and identifying an individual sulfa drug in a sample. Finally, we are collaborating with Dr. Bruce Hammock, University of California-Davis in an effort to produce anti-dioxin monoclonal antibodies. We have generated a group of immunized animals using conjugates supplied by Dr. Hammock and have studied the response of an existing set of anti-dioxin monoclonal antibodies to these new haptens. We anticipate generating a new set of anti-dioxin monoclonal antibodies in the coming year. **IMPACT/TECH TRANSFER:** Many of these antibodies will have potential for commercial development. We will continue efforts to interest commercial immunoassay kit manufacturers to incorporate these into their products. In some cases a CRADA partner already exists and CRADA partners will be sought for others where appropriate. In all cases the technology will be presented in the scientific literature.

OBJECTIVE B: Development of classical analytical methodologies (e.g., HPLC, GLC) for the analysis of pesticide/drug residues to provide confirmatory testing of the immunoassays.

PROGRESS B: Three HPLC methods have been developed to support our studies on development of immunoassays. First, an extraction and HPLC method for detecting halofuginone in chicken serum has been developed. The method has a detection limit of 1.5 ng/mL, and recoveries of 96-99% were observedin samples spiked at 10 ng/mL. Chickens fed halofuginone at 3 ppm for 10 days resulted in 3.75 ng/mL Halofuginone in the serum. This method was recently published. Secondly, an HPLC method for detection of salinomycin in grain has been adapted for detection of drug residue in chicken liver. The method utilizes post column derivation with vanillin and has a detection limit of 50 to 100 ng/g. Again, this method was recently published. Finally, an HPLC method was developed for detecting furosemide in raw milk. The method, adapted from methods used with serum, has a detection limit of 100 ng/mL and was used to confirm results obtained with the ELISA we developed for this drug.

OBJECTIVE C: Development of molecular imprint resins for extraction, cleanup, and detection of residues in foods.

PROGRESS C: The development of molecular imprints as tools in analytical chemistry is a new and exciting area of study. We have generated a series of molecular imprints including imprints to atrazine, salinomycin, halofuginone, and ceftiofur. We have studied the application of these imprints as stand-alone assays, comparing them to antibody methods, and as chromatographic supports. Preliminary results from these studies have been published and presented at major scientific meetings. We anticipate future studies aimed at refining the methods for producing and optimization of the conditions for applying molecular imprint resins to analysis of residues in foods.

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DEVELOP MULTIPLE RESIDUE IDENTIFICATION METHODS FOR TESTING FOOD ANIMAL TISSUES

ARS Contact Persons:
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CRIS Number: 1265-42000-001
Termination Date: January 1997
FSIS Number: I-2

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OBJECTIVE A: Develop simple and rapid physical-chemical procedures for detection and confirmation of antibiotic residues in animal products at levels of concern to regulatory agencies, and verify methods using incurred residues from treated animals.

PROGRESS A: Studies were completed on development of a rapid method for determination of tetracycline antibiotics in tissues. Tissues were blended with water and extracted/deproteinized with 1N HCl and acetonitrile. The resulting filtrate was evaporated, filtered and analyzed directly by ion-pairing HPLC. The extraction procedure used less than 20 ml of acetonitrile/sample. The procedure is rapid with recoveries of 75-90% depending on the compound and substrate. The tetracyclines were stable under the conditions used.

Studies were continued on multiresidue determination of a-lactam antibiotics and major metabolites in tissues. The automated HPLC cleanup was used successfully for separation and cleanup. Fractions were collected and tested for antimicrobial activity by the Delvotest P. Fractions testing positive were further analyzed by HPLC. Residues were confirmed by treating a replicate with a-lactamase prior to analysis.

Ceftiofur was identified as a possible UMI in tissue samples. Studies by the Upjohn Co. demonstrated that ceftiofur was rapidly converted to metabolites when injected into animals. The principle unbound metabolite was desfuroylceftiofurcysteine (DFCC). A standard was obtained from Upjohn Co. and a method for determining the compound in tissues was developed. This was used for determination of residues in tissues of a calf dosed by Dr. Sandra Buckley at Texas A&M. Our results confirmed that the major metabolite was DFCC which was concentrated mainly in the kidney. DFCC was also identified in one UMI sample from FSIS. Cloxacillin and desacetylcephapirin, the major metabolite of cephapirin, are other possible cephalosporin-like UMIs. Cloxacillin was also identified in one UMI sample from FSIS.

IMPACT/TECH TRANSFER: Staff members from the FSIS Eastern and Midwestern Laboratories were given hands on instruction in the tetracycline method and the automated HPLC cleanup method for a-lactam residues. The HPLC a-lactam method is a key part of a joint FSIS-ARS study on identification of unknown a-lactams in tissue which were detected by screening tests. The method has also been used for determination of ceftiofur and metabolites in tissues of a treated calf in support of Dr. Sandra Buckley who is developing an immunoassay for these compounds.

PUBLICATIONS:

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Moats, W. A. and R. Harik-Khan. 1995. Determination of cephapirin and desacetylcephapirin in beef and pork tissues. 210th American Chemical Society Meeting, Chicago, IL, Aug. 20-24. AGFD 9079 (Abstract)

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DEVELOPMENT OF METHODS OF ANALYSIS FOR RESIDUES

ARS Contact Persons:
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Termination Date: December 1997
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OBJECTIVES: Develop quantitative multiresidue methods of analysis for chemical residues in meat and other agricultural products suitable for regulatory purposes, emphasizing the use of biosensors, chromatography, fluorescence, ion-trap mass spectrometry and supercritical fluid extraction to maximize recoveries of analytes while minimizing or eliminating matrix interferences.

PROGRESS: Determining Ten Synthetic Pyrethroids in Ground Meat Using Ion-Trap Mass Spectrometry and Electron Capture Gas Chromatography. Conventional extraction procedures using acetonitrile were compared with supercritical fluid extraction(SFE) using CO_2 for determining 10 pyrethroids in fortified meat and lettuce samples. GC/ITMS proved satisfactory for analysis of lettuce while EC-GC proved more suitable for residue analysis of meat samples. Non-volatiles can accumulate on the capillary column in the GC-ITMS mode shortening its lifetime after multiple injections. The improvement of multiresidue methods for monitoring of synthetic pyrethroids in raw agricultural commodities aids in the establishment of new tolerances under registration and reregistration. **IMPACT/TECH TRANSFER:** The techniques described offer opportunities for improvement of multiresidue methods for analysis of synthetic pyrethroids in meat and vegetables.

Supercritical Fluid Extraction (SFE). A method of multiresidue analysis using SFE and GC/ion trap MS detection for 75 pesticides was developed and tested in fruits and vegetables. A mixture of 2:1 $MgSO_4 \cdot H_2O$:Hydromatrix as the drying agent was found to improve recoveries of polar analytes while maintaining good sample consistency for SFE. The procedure uses 2 g subsamples taken from homogenized 50 g representative samples and requires only 1 mL of acetonitrile to remove the extracted residue from the C-18 sorbent trap. The sample extract can be directly injected in GC/ITD without further clean-up. A sample can be prepared, extracted, analyzed, and confirmed for 75 pesticides within 90 minutes. A pilot study to test equivalency of the SFE method versus traditional methods in the Pesticide Data Program will begin in October 1995.

For meats and other lipid-containing matrices, further clean-up is required when using CO_2 as the extraction solvent. Dr. Jerry King and his research group (USDA NCAUR, Peoria, IL) have demonstrated the lower solubility of lipids in supercritical CHF_3 (fluoroform). After consultation with Dr. King, a cylinder of fluoroform was obtained to investigate the SFE of multiple pesticides in meat, poultry, and eggs. **IMPACT/TECH TRANSFER:** The information gained from studies performed on produce will be very helpful in adapting the multiresidue method to meats.

Accelerated Solvent Extraction (ASE). In July 1995, a Dionex ASE 200 alpha instrument was obtained for research. ASE is an automated technique that extracts samples using liquid solvents at high pressure (≤3000 psi) and temperature (≤200°C). Samples are contained in 11, 22, or 33 mL vessels, and extracts are collected in 40 or 60 mL vials. The increased pressure and temperature, and reduced sample size, reduces the amount of solvent and time required for extraction versus traditional soxhlet methods. The ASE instrument is automated in similar fashion to commercial SFE instruments which

perform extractions sequentially. Unlike SFE, traditional soxhlet or other organic-solvent-based methods can be easily modified for ASE by using the same solvent. However, ASE is not as selective as SFE in separating analytes from matrix components, and clean-up is required for traditional methods of analysis.

Studies were performed using ASE to extract 2,4-D and benomyl (detected as carbendazim) from meat and eggs. ELISA with Ohmicron Rapid Assays was used for analysis which required no clean-up of diluted extracts. Calibration results of assays comparing meat extracts versus water often showed no difference in response. Comparisons of ASE conditions with water and methanol at different pH were made. At all conditions, a definite difference was observed in the ELISA responses of blanks from samples fortified at tolerance levels. The reproducibility of the ELISA response was very good, especially with the benomyl kit (due to the lower detection limit), but quantitation reproducibility was poor due the nature of the inversely proportional, semi-logarithmic calibration plots associated with ELISA. ELISA is a useful screening tool with a low rate of false negatives, and samples extracts found to be positive can be analyzed by a traditional approach.

Flow Immunosensor. The flow immunosensor works by immobilizing an antibody on a column of glass beads, incubating the antibody with an antigen-dye conjugate, and upon injection of true antigen in a flowing system, the antigen-dye is displaced and measured by fluorescence downstream. A prototype flow immunosensor was developed for alachlor, but FSIS (Athens, GA) findings have shown that alachlor does not occur in meat. Further research on alachlor has not been pursued. Dr. Larry Stanker's group (USDA ARS FAPRL, College Station, TX) supplied an antibody for fumonisin for use in the flow immunosensor. Initial experiments were performed, and detection limits were too high (0.3 µg/mL) to warrant further study.

PUBLICATIONS:

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N.Aharonson, S. J. Lehotay, and M..A. Ibrahim. 1994. Supercritical Fluid Extraction and HPLC Analysis of Carbendazim and Thiabendazole in Potato, Apple, and Banana. J. Agric. Food Chem. 42:2817-2823.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO RESIDUE DETECTION AND NUTRIENT ANALYSIS

ARS Contact Persons:
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OBJECTIVE A: Develop supercritical fluid- and conventional liquid-based size exclusion sample cleanup methods.

PROGRESS A: Efforts this past year have focused on the refinement of using different eluents, both liquid as well as supercritical carbon dioxide, in place of methylene chloride in the size exclusion chromatographic cleanup of meat extracts containing pesticide residues. A considerable number of pesticide types have been run and their retention characterized on a commercial column (Jordi Gel), which is resistant to high pressures and mixtures of SC-CO_2 with different organic liquids. Retention patterns for SC-CO_2 mixtures with p-dioxane, ethyl acetate, and tetrahydrofuran give more than adequate separation of the pesticides from the fat peak, and result in further separation of the pesticide classes, similar to that found when using neat methylene chloride as the column eluent.

Recently, neat methyl-t-butyl ether, because of its lower toxicity, has been investigated as a substitute for methylene chloride. Separation of pesticides from fat is excellent and pesticide retention patterns on Jordi Gel previously found for other eluents are maintained using methyl-t-butyl ether (MTBE). Fat recovery studies with the MTBE/Jordi Gel system have been 100%, at 0.5-1.0 g loadings. This research has been accomplished with the assistance of a foreign visiting scientist, Dr. Fabio Favati, of the University of Potenza in Italy.

Currently we are attempting to determine pesticide recovery levels and fat contamination levels using mandated GC/ECD analysis. The selected pesticides comprise those found at levels in samples furnished to us by the FSIS Western Laboratory as part of a RFP proposal to commercial companies. This study is being undertaken with the collaboration of Marvin Hopper of FDA's Total Diet Research Center in Lexena, KS.

OBJECTIVE B: Provide training and information to scientific community on supercritical fluid techniques.

PROGRESS B: A successful symposium entitled, "New Approaches to Sample Cleanup and Extraction" was presented by J. King at the 1994 Pittcon Conference in New Orleans in 1996. Among the speakers were Jeff Brewster of ERRC. Janet Snyder organized a very successful symposium at the 1995 American Oil Chemists Society Meeting in San Antonio, Texas. Similarly, Jerry King and Scott Taylor have organized a symposium for the 109th International AOAC Meeting in Nashville, TN entitled, "Advances in Supercritical Fluid Extractions and Microwave Extraction". Topics to be covered include SFE for pesticide analysis (S. Lehotay-BARC) pressurized liquid extraction (B. Richter - Dionex Corp.), and a comparison of various extraction methods by D. Raynie, Procter & Gamble.

IMPACT/TECH TRANSFER: J. King is currently organizing the 7th International Symposium on SFC and SFE to be held in Indianapolis, IN on March 31 - April 4, 1996. Due to the meeting's location, there will be a particularly strong emphasis on drug and food analysis by SFC and SFE. R. Ellis (FSIS), S. Lehotay (ARS), R. Maxwell (ARS), and S. Taylor (ARS) will be making presentations. J. King participated as the keynote lecture at the Mid-Atlantic Regional AOAC Meeting held in Lancaster, PA this past year which covered the topic of supercritical fluid extraction. The large turnout for this meeting (75) was comprised of many scientists from regional state laboratories as well as laboratories in the Washington, D.C. area. J. King is also co-lecturer with Professor Larry Taylor of the prestigious short course, "Supercritical Extraction/Fractionation/Chromatography" which was offered by the American Chemical Society twice this past reporting year. King also filed an AOAC associate referee's report on the SFE of meat products for general referee, Dave Soderberg, during this past reporting period.

PUBLICATIONS:

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King, J.W. and Nam, K. 1995. Coupled enzyme immunoassay (EIA) with supercritical fluid extraction (SFE) in Residue Analysis in Food Safety: Applications of Immunoassay Methods, eds. R.C. Beier and L. Stanker, American Chem. Soc., Wash., D.C., (Accepted)
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Taylor, S.L. and King, J.W. 1995. SFC for sample preparation and residue and nutrient analysis in foods. Proceedings of the 17th International Symposium on Capillary Chromatography and Electrophoresis, Wintergreen, VA, pp. 176-177.

DOSED TISSUES AND FLUIDS (INCURRED RESIDUES) OF HERBICIDES, INSECTICIDES AND OTHER CHEMICALS THAT MAY CONTAMINATE MEAT AND POULTRY

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CRIS Number: 6202-42000-007
Termination Date: March 2000
FSIS Number: I-2

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OBJECTIVE A: To produce food-animal tissues and body fluids containing specified levels of incurred residues of veterinary drugs, pesticides, or other chemicals for use by FSIS in the development and validation of analytical methods.

PROGRESS A: Five studies involving incurred drug residues in animal tissues and fluids were completed: 1) Phase II of the spectinomycin study in broilers was completed in 4/95. Liver, kidney and muscle tissues and plasma were sent to FSIS-ERRC. 2) A beef calf was treated with ceftiofur. Muscle, kidney and serum were collected and sent to USDA-ARS-BARC. A duplicate set of tissues and fluids were kept at FAPRL for later use. 3) Sulfonamide (sulfamethazine, sulfadimethoxine and sulfathiazole) residues were produced in porcine tissues and fluids in 7/95. Muscle, liver, kidney, serum and urine were collected. Muscle and liver samples were sent to USDA-FSIS Eastern Laboratory and the remaining tissues and fluids were retained at FAPRL for future use. 4) Also in 7/95, a beef calf was treated with 5 mg/kg enrofloxacin for 5 days to produce desethylene ciprofloxacin (a metabolite) residues. Muscle, liver, urine, and plasma were collected and sent to the USDA-FSIS Midwestern Laboratory. 5) In 8/95, Ceftiofur residues were produced in the serum and milk of three dairy cows. Cows were treated for 5 days at 2.2 mg/kg. A set of milk and serum samples were sent to USDA-ARS-BARC and a second set retained at FAPRL for analysis.

IMPACT/TECH TRANSFER: Reports detailing each incurred project were prepared and forwarded along with the samples to the FSIS or the FSIS designated agency.

PUBLICATIONS:

None

IMMUNOCHEMICAL BIOSENSORS

ARS Contact Person:
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CRIS Number: 5325-42520-001
Termination Date: January 1997
FSIS Number: I-2

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OBJECTIVE A. Apply immunochemical and biosensor methods to quantify components of foods which influence the safety and healthfulness of the food supply.

PROGRESS A. This subproject builds on the previous work done to develop screening assays for benzimidazole antihelmintics, including thiabendazole, fenbendazole, oxfenbendazole, oxibendazole, cambendazole, and albendazole. A Cooperative Agreement, initiated in November 1994 with Millipore Corporation (Bedford, MA), has facilitated commercialization of our thiabendazole method as part of the EnviroGard immunoassay kit product line. Collaboration with Millipore's subsidiary, ImmunoSystems, Inc. (Scarborough, ME) is continuing under this agreement. An ELISA based on our multiresidue method for benzimidazole residues is now in use at the Central Veterinary Laboratory, Surrey, UK, and assay materials for the thiabendazole ELISA have been provided to the Department of Agriculture for Northern Ireland. Cooperative work has been undertaken recently with the National Food Centre, Dublin, Ireland and the Laboratoire de Pharmcologie-Toxicologie, INRA, Toulouse, France under a European Union Agro-Industrial Research Project. One objective of the project is to characterize the nature and toxicity of "bound residues" of certain veterinary drugs, including thiabendazole, fenbendazole, and mebendazole. We are preparing antibodies which could be used to identify bound residues in situ or to isolate and identify the bound residues after hydrolysis. Studies have been undertaken to determine whether layers of immobilized antibodies can be effectively patterned by photolithography and oxygen plasma exposure. Immobilized antibody layers were protected from denaturation by coating with a polyvinyl alcohol solution prior to spin coating with photoresist. Selective UV exposure through a mask was followed by development of the exposed resist. The final step was exposure to oxygen plasma. This procedure proved successful in selective deactivation of regions of the antibody layer. Current work is focussed on estimating the smallest patternable dimension.

IMPACT/TECH TRANSFER: Technology has been transfered in the form of an immunoassay kit now commercially available to FSIS and producers, enabling measurement of residues of thiabendazole, 5-hydroxythiabendazole, and cambendazole. Recent studies of antibody immobilzation provide a general technique for patterning of immobilized protein layers which could be used to fabricate multi-analyte sensors.

PUBLICATIONS:

Flounders, A.W., Brandon, D.L., and Bates, A.H. 1995. Immobilization of thiabendazole-specific monoclonal antibodies on silicon substrates by aqueous silanization. Appl. Biochem. Biotechnol. 50. 265-284.

Brandon, D.L., Binder, R.G., Bates, A.H., and Montague, W.C. Jr. 1995. Competitive ELISA of thiabendazole residues in produce using indirectly immobilized monoclonal antibodies. Food Agric. Immunol. 7. 99-108.

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METABOLISM AND RESIDUES OF SULFADIMETHOXINE AND CLENBUTEROL IN FARM ANIMALS.

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G.D. Paulson

CRIS Number: 5442-32000-006
Termination Date: February 1997
FSIS Number: I-90-6

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OBJECTIVE A: Determine the metabolism, rates and routes of excretion and most appropriate marker residue of sulfadimethoxine in swine.

PROGRESS A: Previous reports have described how eight 25 to 40 kg barrows were dosed with ^{14}C-SDM (60 mg/kg, orally, specific activity approximately 1000 dpm/g) and two animals each were sacrificed at 12, 24, 48, and 72 hours after dosing. Tissues were collected, ground and the disposition of total radioactivity was determined by sample oxidation. Urine and feces were collected at 6 and 12 hours post dosing and then every 12 hours until sacrifice. Details of the tissue disposition and elimination of ^{14}C-activity have been previously reported. Studies completed during the past year demonstrated that ^{14}C-SDM accounted for 24% (liver) to 66% (adipose tissue) and the N^4-acetyl derivative of SDM (N^4-Ac-SDM) accounted for 10% (skeletal muscle) to 35% (kidney) of the total ^{14}C-activity in tissues 12 hours after dosing with ^{14}C-SDM. The N^4-glucose conjugate of SDM (G-SDM) was a major ^{14}C-labelled fraction in skeletal muscle (21% of total ^{14}C-activity) and liver (28%) but was not detected in adipose tissue or kidney. The N^4-glucuronic acid conjugate of SDM (GA-SDM) was a minor metabolite in urine and kidney, but was not detected in other tissues. Desamino-SDM was a minor metabolite in the kidneys. ^{14}C-Labeled fractions isolated from 0-6 hour urine included N^4-Ac-SDM (82%), SDM (3%) and GA-SDM (6%). A minor metabolite (0.7% of total ^{14}C-activity) was isolated from plasma and characterized by ^{1}H-NMR and mass spectrometry as the sulfate ester of 3-hydroxysulfadimethoxine.

IMPACT/TECH TRANSFER: These studies have demonstrated that the metabolism of sulfadimethoxine is much more complex than earlier studies (conducted without radiotracer) suggested. The information will be useful to FSIS and to FDA in relation to establishing marker compounds and withdrawal periods from the drug..

OBJECTIVE B: Determine the metabolism, rates and routes of excretion and most appropriate marker residue of clenbuterol in cattle.

PROGRESS B: Two Holstein calves were given a single oral dose of ^{14}C-labeled clenbuterol (^{14}C-CB) and urine (0-48 hours after dosing), feces (0-48 hours after dosing) and tissues (48 hours after dosing) were collected and analyzed for ^{14}C- activity. The average total urine collection accounted for 41.5% and the feces 2.4% of the ^{14}C-activity given to the animals. Forty-eight hours after dosing, the animals contained an average of 52.3% of the ^{14}C-activity administered as ^{14}C-CB. The ^{14}C-activity remaining in the animals was widely distributed; however, the lungs, liver and kidneys were the tissues with the highest residues. The skeletal muscle and adipose tissues and blood were the tissues with the lowest concentrations of ^{14}C-activity. Two ^{14}C-labeled fractions were isolated from the urine and identified as ^{14}C-CB and a glucuronic acid conjugate of CB (the site of glucuronic acid conjugation was not determined). Studies are currently underway to isolate and identify other ^{14}C-labeled metabolites in the urine. Studies to characterize the ^{14}C-labeled compound(s) in the tissues will also be conducted.

IMPACT/TECH TRANSFER: This information is needed to provide the basis for appropriate monitoring of legal as well as potential illegal use of clenbuterol in animal agriculture. These studies have demonstrated that the metabolism of clenbuterol is complex and that the rate of depletion of clenbuterol-related residues is relatively slow. This is of importance to FSIS and to FDA as it relates to establishing the withdrawal time for the drug.

PUBLICATIONS

Adams, P. E., V. J. Feil, and G. D. Paulson. 1995. Metabolism of 14C-sulphadimethoxine in swine. Xenobiotica. (Submitted).

DIOXIN RESIDUES IN FOOD PRODUCING ANIMALS

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CRIS Number: 5442-42000-001
Termination Date: February 1997
FSIS Number: N/A

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OBJECTIVE: To identify and quantitate residues of chlorinated dioxins and furans in beef and in animal feeds, in particular, forages. To devise strategies for minimizing the occurence of these residues in order to minimize the impact on the consumer.

PROGRESS A: The United States Environmental Protection Agency (EPA) has designated beef as a major contributor to the human dioxin burden because animals grazing on forage contaminated by fallout from burning processes would store these lipophilic materials in adipose. A statistical survey of chlorinated dioxin and furan levels in beef consumed by the U.S. population was conducted jointly by the EPA and the United States Department of Agriculture. The survey was designed to provide data on the dioxin burden that the population receives from consumption of beef. Details on feeding regimes, age and geographical sources of the feed consumed by the animals being surveyed were generally not available. We selected experiment stations in the following states: CT, FL, GA, HI, ID, IN, MT, NE, NM, ND, OK, OR, PA. The collections are being made as part of normal management operations and will be made over approximately a two year period. Ultimately, most stations will provide us with samples from a sufficient number of animals to allow us to determine animal variations from a given site as well as geographical variations. Samples collected are back fat, perirenal fat, ribeye muscle, liver and serum from healthy bulls, 2 years old and older; females, 1-2 years old, 4-6 years old, and 8 years or older. We plan to do all of the matrixes from only a sufficient number of animals to establish relationships. These preliminary studies on 20 perirenal fat samples generally show a preponderance of the higher chlorinated congeners with a few animals showing non-detect levels for all congeners and a few animals from a given site showing levels substantially higher than the average for that site. The congener profile should help identify the source of dioxins available to the animals.

PROGRESS B: The dioxin interaction with various mammalian carrier proteins using 1,2,7,8-; 1,3,7,8-; and 1,4,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were studied. Parent 1278-TCDD was not detected in the urine. Binding of ^{14}C from 1278-TCDD to albumin was believed to be the result of metabolites of 1278-TCDD. Of the dose 14.3% was excreted in the urine in 72 h with about 3% (1% each 24 h) bound to albumin. Unbound metabolites accounted for 8.0% of the dose in the first 24 h period, then dropped to 3.3% in the 24-48 h period and were not detected in the 48-72 h period. ^{14}C-1278-TCDD metabolites were not extractable from the albumin with common organic solvents indicating possible covalent bonding between the 1278-TCDD metabolites and albumin. ^{14}C-1278-TCDD metabolites were not bound to alpha 2u-globulin in the urine. Binding of ^{14}C residues to kidney and liver proteins represent most of the ^{14}C in these tissues (91% and 96%, respectively). Larger proteins bound most of this ^{14}C. Albumin, the most prevalent of these proteins, is believed to bind most of this ^{14}C, as was the case in the urine. Considerable binding of ^{14}C residues to the 14 kDa protein isolated from kidney and liver tissue was also observed (12% and 4.6% of the ^{14}C in the tissue, respectively). Unbound ^{14}C in 0-24 h urine was characterized by beta-glucuronidase or aryl sulfatase enzyme hydrolysis and mass and NMR spectrometry to be the beta-glucuronide-sulfate ester diconjugate of catechol (35% of the ^{14}C in 0-24 h urine).

IMPACT/TECH TRANSFER: These studies show that metabolites from 1,2,7,8-TCDD can bind to transport carrier proteins in animals which may explain their transport and storage in animals.

PROGRESS C: The metabolism of the less toxic 1,2,7,8-tetrachloro-dibenzo-p-dioxin (1278-TCDD) was initially selected for study because more dioxin could be fed, allowing for greater mass from which to isolate metabolites. [UL 7,8 ring ^{14}C] 1278-TCDD was synthesized and given in peanut oil by gavage (0.5 ml of oil, 2 mg of dioxin, 9.4 microcuries per rat) to six male Sprague-Dawley rats (weights 235 to 254 g). Feces and urine were collected for three days. Over eighty percent of the ^{14}C was recovered in the feces, most in the first two days, and 14.3% was recovered in the urine, most in the first day. Two urinary metabolites were characterized by the ^{1}H-NMR spectrum and the negative ion FAB mass spectra to be the beta-glucuronide/ sulfate diconjugate of 4,5-dichlorocatechol and the sulfate conjugate of 4,5-dichlorocatechol. Metabolic alteration of xenobiotics is generally assumed to lead to detoxification and elimination of harmful compounds. There are many exceptions to this generalization however, most recent and relevant to dioxins being the methylsulfone and hydroxylated metabolites of PCBs and DDT. Metabolic studies with a series of differently-substituted dioxins combined with NMR spectroscopic identification of biogenic isomers may explain some of the toxicity of these compounds. In the case of ^{14}C 1,3,7,8-TCDD in the rat, most of the dose is excreted in the feces (94% within 72 hrs) and only trace amounts appear in the urine (1.7%). Analysis by HPLC showed 25% of the radioactive material to be parent compound and 75% to be a more polar metabolite. Further chromatography with TLC resolved two isomers that had identical mass spectra and GLC or HPLC retention times but by their NMR spectra were identifiable as 2-OH-1,3,7,8-TCDD and the NIH shift product 1-OH-2,3,7,8-TCDD. If only chromatographic behavior and MS spectra had been used to identify these metabolites as has been the case in previous studies, it would not have been possible to distinguish between them. That the 2-OH metabolite is also a possible product of the most toxic known dioxin, 2,3,7,8-TCDD demonstrates the interrelatedness, complexity, and toxicological relevance of isomer identification problems in this series of compounds.

IMPACT/TECH TRANSFER: These studies show that 1,2,7,8- and 1,3,7,8-TCDD are metabolized in mammalian systems which involve conjugation to form beta- glucuronides and sulfate esters. In the case of 1,2,7,8-TCDD, the 4,5-dichloro catechol is formed and in the 1,3,7,8-TCDD, a NIH shift occurs, which produces a 2-OH metabolite, a possible product of the toxic 1,2,7,8-TCDD.

PUBLICATIONS:

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PHARMACOKINETIC MODELS

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CRIS Number: 1265-32000-046
Termination Date: September 1997
FSIS Number: I-89-1

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OBJECTIVE A: To develop mathematical models of the pharmacokinetics of TCDD related compounds in beef and dairy animals.

PROGRESS A: The major thrust of this project addresses questions concerning the sources and transport of TCDD (2,3,7,8-tetrachloro-p-dibenzodioxin) and related compounds to human foods. It is generally concluded that animal foods are the major route of exposure because the dioxin-like compounds are lipophilic, and depending on the number and positions of chlorine substitution, resistant to metabolic degradation. Realistic transport models are required for human exposure assessments because literature on the behavior of dioxins in farm animals is scant and often lacks the ancillary physiological information and because dioxin analyses are expensive. The coplaner PCBs (polychlorinated biphenyls) are a class of compounds with dioxin-like activity that are less well characterized than the dioxins and furans. A cooperative agreement was established with the Ohio Agricultural Research and Development Center in order to utilize archived samples and data from a dairy cow experiment with the Aroclor 1254 PCB mixture. Selected samples of milk, adipose tissue, plasma and feces are being analyzed on an individual congener basis. This will provide the basis for establishing pharmacokinetic parameters of coplaner PCBs in dairy cattle and the parameters of the broad array of congeners will allow inferences to be drawn concerning the relationship between physical properties and the transport of residues to milk and tissue. The work is in progress and definitive results are not available. The congener distribution profiles of the FSIS-EPA beef survey and published food surveys have been reviewed in an attempt to identify the potential animal and human sources of exposure. The major conclusion to be drawn from these efforts is that the residue burdens of the more highly chlorinated dioxin in the human population can not be accounted for by the concentrations in foods and the generally accepted biotransfer factors for these congeners. Similar conclusions can be drawn when comparing the profiles of animal products with identified environmental sources. Close interaction with the FSIS-EPA meat sampling project and the ARS-Fargo metabolic project will be maintained and the results will be used to validate or modify the models. **IMPACT/TECH TRANSFER:** There are either unidentified sources of exposure to dioxin or the data base on bio-transfer factors is inadequate. Ongoing research at ARS-Fargo and other research centers should provide improved estimates of biotransfer.

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DETERMINING TEMPERATURE TO WHICH PRODUCTS HAVE BEEN COOKED

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OBJECTIVE A: Determine Acid Phosphatase (ACP) activity in model cooked poultry breast, thigh, and a 50/50 blend of breast and thigh for both marinated and non-marinated meat.

PROGRESS A: Results of a cooperative study involving 3 laboratories (Agricultural Research Service, Dutch Quality House, and the Food Safety and Inspection Service, Eastern Laboratory) have been summarized and submitted to AOAC International for review under the AOAC Peer-Verified Methods Program. Results of the study showed that FLUOROPHOS ACP activity could be used as a 15-20 minute analytical method to determine end-point temperature (160 °F/71.1 °C) in selected cooked poultry products, including marinated and nonmarinated broiler breast and thigh, and a 50-50 blend of breast and thigh. Statistical analysis indicates that the ACP method has a mean -1.1 F 95% confidence limit below the 160 °F/71.1 °C FSIS regulation for the tested poultry meat products.

IMPACT/TECH TRANSFER: The method has been formally submitted to FSIS Chemistry Division for review. It is envisioned that the method will be used to verify compliance of certain poultry products having been heat processed to 160 °F/71.1°C. Further, the method is being adapted by the cooperating commercial manufacturer as a quality assurance/HACCP method for a commercial poultry processor.

OBJECTIVE B: Determine catalase (CAT) activity in model cooked ground beef and pork sausage for patties.

PROGRESS B: Catalase activity in model system ground beef and pork was determined on samples cooked from 60 to 71.1 °C. One gram samples of ground round (4% immersed in an ice-water bath (0-1 °C) to quick-chill the samples and prevent temperature over-run. Samples retained high CAT activity through 90, 60, and 45 sec at 65, 68.3, and 71 °C; respectively, before showing rapid activity decreases. Ground beef (24% fat) was model cooked according to the time/temperature requirements published by FSIS. Decreasing CAT activity values were significantly ($P<.05$) different among some of the time/temperature groups (151 °F/41 seconds, 153 °F/26 seconds, 155 °F/16 seconds), but not different between 155 °F/16 seconds and 157 °F/10 seconds. Freezing ground beef prior to cooking caused a reduction in CAT activity with significant differences at 153 °F/26sec and 157 °F/10 sec.

IMPACT/TECH TRANSFER: Current research findings indicate that this method could be used by the FSIS Food Chemistry Labs, and state or local health departments to verify compliance of beef pattie and sausage pattie products (157 °F/10 second) as required by the Pattie Product Regulation. Further, the method could be used in fast food quality assurance programs to verify FSIS/FDA regulations.

OBJECTIVE C: Determine the potential of residual glutamic-oxaloacetic transaminase (GOT) activity in thermally processed beef (cured and uncured) and poultry as an indicator of end-point temperatures (EPT).

PROGRESS C: Beef samples were acquired from the University of Georgia and the University of Florida so that color and residual GOT activity in beef logs fabricated and heat treated to simulate commercial products imported from South America could be measured. A Cooperative Research and Development Agreement was entered into with a private company so that GOT activity in commercially fabricated cured beef logs could be determined. Beef samples from the two universities were separated into cuts (top sirloin, top round, sirloin tip, gooseneck round, and chuck roast) and animal age (18, 36, and 60 months). Color retention and GOT activity were measured after heating to desired EPT. Initial results of fabricated logs heat treated to 69, 74, and 79 °C in a 100 °C water bath indicate progressive declines in residual GOT activity, but retention of red color for both meat tissue and juices at all of the EPT evaluated. Variations in objective color values (L, a, b) were noted by sampling different locations within the logs. In general, higher a values (measure of redness) were found at the geometric center of the logs. A study is now underway to evaluate color and GOT activity in logs heated to an EPT of 84 °C.

Preliminary studies were conducted with commercially fabricated cured beef logs cooked to EPT of 71, 77, and 85 °C. Results indicate measurable differences in GOT activity; however, the activity declines were not consistent with increasing temperature, indicating either interference with the GOT measurement by curing additives, or improper labeling of the logs in the commercial facility prior to shipment to the lab. Ongoing discussions with the cooperator are focusing on heating conditions, and accurate labeling of the beef log samples. Use of residual GOT activity as an indicator of EPT in processed chicken is being tested on commercially prepared products. Samples of whole muscle, formed patties, strips, marinated white meat, diced thigh meat and a 50/50 blend of white/dark meat from a commercial further-processor were evaluated for residual GOT activity. Estimated EPT based on residual GOT activity compared favorably with measured EPT of 71.1 and 73.9 °C for the white and dark meat, respectively. Estimated EPT by the GOT procedure were 74 °C.

IMPACT/TECH TRANSFER: Measuring residual GOT activity appears to be an accurate way to predict EPT in processed poultry products. This objective marker may be used to monitor and/or verify heating, the most critical control point of the HACCP concept.

OBJECTIVE D: Evaluate factors which affect cooked poultry meat color.

PROGRESS D: Color is commonly used to assess doneness in cooked poultry meat. Over the past several years, there have been reports of fully cooked poultry meat exhibiting an undercooked appearance (red to pink color). Two experiments were conducted to identify some processing factors that might contribute to variation in cooked poultry meat color. In the first experiment, turkey Pectoralis major muscles were harvested, at various post mortem times, ground and cooked to an EPT of 72 °C. It was noted that if muscles were harvested and processed before resolution of rigor mortis (prerigor), they were more likely to exhibit a pink color after cooking than if they were

harvested and processed after the resolution of rigor (postrigor). This difference in color was ascribed to the protective effect of high pH in prerigor muscle on the pigment myoglobin. In the second experiment, the effects of electric stunning time used to immobilize broiler chickens and the addition of sodium tripolyphosphate (STPP) to Pectoralis major muscles on cooked meat color were determined. All of the muscles were cooked to an EPT of 72 °C. Subjecting broilers to electric stunning times ranging from 2 to 10 seconds had no effect on subsequent cooked meat color, but STPP treated muscles were less red and darker than controls.

IMPACT/TECH. TRANSFER: The results indicate that processing variables such as use of prerigor muscle to fabricate products can affect color of cooked poultry breast meat leading to erroneous conclusions if doneness is assessed strictly on the basis of color.

OBJECTIVE E: Develop methodologies to characterize detrimental traits such as rancidification and putrification.

PROGRESS E: The scope of the CRIS project was broadened this year to include a request from FSIS to develop a method to evaluate rancidity and detect putrefactive spoilage (Food Safety Research: Current Activities and Future Needs, Nov. 23, 1994). Research has been initiated to develop methodologies to characterize the volatile isolates from meat products that have been developed through improper storage temperatures. Emphasis is being placed on development of rapid analytical procedures to detect staleness, rancidification or putrification. Initial research using GC-MS analyses of volatiles isolated from the headspace of packaged products by solid-phase micro extraction procedures are providing profiles that are indicative of spoilage in beef and poultry products. Modification and refinement of these procedures may make it possible for regulatory agency personnel to detect character notes specific to the early stages of rancidification and spoilage in meat and poultry products.

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METHODS TO DETERMINE THE TEMPERATURE TO WHICH PRODUCT HAS BEEN COOKED

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CRIS Number: 1265-41440-001
Termination Date: February 1997
FSIS Number: I-5

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OBJECTIVE A: Identify mechanisms and develop control procedures to prevent inconsistent cooked meat color-cooked meat temperature relationships.

PROGRESS A: Government agencies have suggested that consumers cook beef patties until they reach 160 °F (71 °C), when no pink/red color exists in the meat and the juices run clear. It has been thought that these situations have all occurred at 71 °C. We evaluated 17 commercially processed beef patties (most low fat) and found eight of the products retained red color at 71 °C. The red color was due to undenatured myoglobin and in some cases elevated pH in the product. One-year of frozen storage produced additional red color. Cooking to 81 to 87 °C was often necessary to eliminate the red color. Additional studies were performed using normal and high pH beef and beef from a meat recovery system. Use of meat from the lean meat recovery system resulted in a high pH, but did not produce the red cooked color normally associated with high pH meat. Conditions during processing of meat from meat recovery systems may be responsible for the increased heat sensitivity of myoglobin in this material. Three studies were conducted to evaluate the color of patties cooked immediately after processing, after freezing and after freezing and thawing. Patties cooked from the frozen state were less red than those cooked immediately after processing. Premature browning, the phenomenon where the product appears well-done at temperatures lower than 71 °C, only occurred in thawed patties. After 24 hours of thawing, patties appeared well- done at 65 °C. Studies were conducted to determine the degree of variability in cooking properties when selected degrees of control were exerted in the cooking process. Cooking of patties for a constant time period, that on-the-average produced 71 °C internal patty temperatures, resulted in 9.0% of the patties not reaching 68 °C and 1.3% not obtaining 60 °C internal temperature.

IMPACT/TECH TRANSFER: Cooked patty color is an inaccurate indicator of cooked meat temperature. Consumers should use accurate temperature measuring devicesinsure safety. Premature browning clearly implies a safety risk. If patties are thawed before cooking, there is a much higher incidence of premature browning. Several meetings were held with representatives of FSIS to transfer this information. The information was of great importance to FSIS for their use in developing "Backgrounders" for consumers on how to cook hamburgers.

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LIPID OXIDATION PRODUCTS IN FRYING OILS AND FOODS AS POTENTIAL HEALTH HAZARDS

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OBJECTIVE A: To study the effect of frying variables such as frying time and temperature, oil type, presence or absence of antioxidants on the possible production of mutagenic substances in repeatedly used frying oils.

PROGRESS A: The volatile constituents of used frying oils obtained from local food processing plants were isolated by simultaneous distillation-extraction (SDE) and fractionated by silica gel chromatography. The isolates were analyzed by capillary gas chromatography and combined capillary gas chromatography-mass spectrometry (GC-MS) resulting in the identification of 140 compounds. The major constituents identified were 1-pentanol, hexanal, furfuryl alcohol, (E)-2-heptenal, 5-methylfurfural, 1-octen-3-ol, octanal, 2-pentylfuran, (E)-2-octenal, nonanal, (E)-2-nonenal and hexadecanoic acid. Since the determination of frying oil lifetime is one of the goals of this project the identification of these marker compounds which increase during frying is potentially useful since their level may be an indication of oil quality. We synthesized a series of 4- and 5-oxoaldehydes and four of these oxoaldehydes, 4-oxohexanal, 4-oxooctanal, 4-oxononanal and 4-oxodecanal were identified for the first time in used frying oil. Oxoaldehydes have been proposed as precursors of 2-alkylfurans which have potential anticancer effects. In a model reaction 4-oxononanal was refluxed in hexane for 40 days and only trace amounts of 2-pentylfuran were produced, suggesting that it is not a major precursor of the furan. In June of this year we wrote a revised research plan with emphasis on determining the effect of frying variables on the chemical properties of the oil. Several methods such as determination of total polar components, measurement of polymerized triglycerides, iodine value, level of thiobarbituric acid reactive substances (TBARS) are being used to assess heat damage of the oils under defined conditions. Toxicological testing such as the Ames assay are in progress and whole oil samples and polar and non-polar oil fractions will be tested. Mutagenic activity of repeatedly used frying oils will be evaluated in relation to their chemical characteristics.

OBJECTIVE B: Develop practical rapid methods for determining when frying oil should be replaced.

PROGRESS B: At present, there are no objective, standardized methods that can be used in food processing plants to determine the extent of thermal and oxidative damage in frying oil. Standard AOCS and IUPAC test methods for measuring the level of oil decomposition are generally very time consuming, labor intensive, require technical expertise and use of solvents which make their use in industry impractical. We have been in contact with a commercial supplier who produces various quick tests that have been developed to provide a rapid means of testing for frying oil damage. We are currently evaluating these test kits for precision, accuracy and reproducibility in relation to standard AOCS and IUPAC methods. We will also evaluate other commercially available test kits and equipment to determine their potential for use in processing plants as well as their limitations, and compare effectiveness in predicting presence of major degradation products. If none of the presently available commercial kits and/or equipment are suitable, other methods of detecting these products will be developed.

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FINAL REPORT: NITROSAMINES IN HAMS PROCESSED WITH ELASTIC RUBBER NETTINGS

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CRIS Number: 1935-42000-016
Termination Date: March 1996
FSIS Number: I-92-1

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OBJECTIVE A: Develop methodology to isolate and quantitate amines and nitrosamines in hams processed in elastic rubber nettings.

PROGRESS A: A solid phase extraction (SPE) method was developed for the determination of 10 volatile nitrosamines, principally N- nitrosodibutylamine (NDBA), in hams processed in elastic rubber nettings. Because of the fat solubility of NDBA, two extraction columns were required. The method uses propyl gallate as a nitrosation inhibitor to prevent any possible artifact formation during analysis. The mean recovery for NDBA fortified at the 10 ppb level was 87.7%, and the minimum detectable level was 1.0 ppb. The repeatability of the method was 1.3 ppb with a coefficient of variation (CV) of 6.2%. The method was compared to two established procedures for NDBA analysis in cured meat products: the mineral oil distillation (MOD) and the low temperature vacuum distillation (LTVD) procedures.

No significant difference was found between the MOD and LTVD methods; these methods were found to yield significantly higher NDBA levels than the SPE procedure. A similar SPE method was successfully used for the isolation of the semi-volatile N-nitroso-dibenzylamine (NDBzA), a nitrosamine resulting from the reformulation of the rubber used to make the elastic rubber nettings. A heated interface between the gas chromatograph and the Thermal Energy Analyzer (TEA) was necessary for the detection of this nitrosamine. Repeatability of the method for NDBzA was found to be 2.1 ppb with a CV of 10.6%. This method was also shown to be free of artifactual nitrosamine formation.

A method to detect dibutylamine (DBA) in ham was developed with a detection limit of 0.1 ppm. The method utilizes a modified TEA, selective for nitrogen (TEA/N) rather than nitric oxide containing compounds. In hams fortified with 0.5 ppm DBA, recoveries of 90.2% +/- 3.4% were obtained. The repeatability of the method was 0.07 ppm with a CV of 6.1%. With minor modifications, the method was applied to dibenzylamine (DBzA) in hams. The repeatability of the method for DBzA was 0.02 ppm, with a CV of 5.7%, and a limit of detection of 0.01 ppm. Recoveries of 85.0% +/- 4.1% were obtained in hams fortified at the 0.5 ppm level. **IMPACT/TECH TRANSFER:** The development of these methods allowed us and others, including FSIS, to study this problem.

OBJECTIVE B: Determine mechanisms for nitrosamine formation in netted hams.

PROGRESS B: Zinc dithiocarbamates are used as vulcanization accelerators in the formulation of rubber. These compounds are the source for the secondary amines that form the nitrosamines in hams exposed to the rubber in the elastic nettings. Studies on the thermal decomposition of purified zinc dibenzyldithiocarbamate (Zn DBzDTC), by a variety of thermal and spectroscopic techniques, were carried out. The Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) showed that 326 degs C was the decomposition temperature of Zn DBzDTC. At this temperature, TGA-FTIR indicated that the dominant decomposition product was CS2; there was no

evidence of dibenzylamine (DBzA). The reason was later determined to be that the temperature of the interface could not be heated sufficiently high to pass this compound into the light pipe. Zn DBzDTC was injected into a GC-MS, whose injection port was heated from 200 to 300 °C in 20 °C increments. The amount of dibenzylamine (DBzA) increased with temperature. Even considering that rubber is formulated with compounds that facilitate accelerator decomposition, the high temperature of decomposition suggests that undecomposed accelerator is still present in the rubber netting, even after smokehouse processing. It also suggests that the amount of free DBzA, present as a contaminant in the crude commercial accelerator, may play a more important role in nitrosamine formation than first thought.

A kinetic study on the formation of NDBzA, from the nitrosation of DBzA by sodium nitrite, was performed in a model system under conditions (temperature, pH) that were similar to those encountered in the commercial production of hams processed in elastic rubber nettings. The nitrosation reaction exhibited first-order kinetics with respect to DBzA, and second-order kinetics with respect to nitrite. The calculated rate constant was 4.7 +/- 0.5 per moles squared per min. The pH profile of NDBzA formation was also determined, with the optimal pH at 3.12. **IMPACT/TECH TRANSFER:** This work gave us information on the mechanisms of formation of NDBzA in netted hams.

OBJECTIVE C: Effect of processing on nitrosamine formation.

PROGRESS C: Studies on the penetration of NDBA and NDBzA and their corresponding amines into hams showed that they could be present at a depth of 1 1/2 inches from the ham surface. The highest concentrations of both amine and nitrosamine were found on the outer surface of the ham with the amine levels being higher. For NDBA and NDBzA, there was a marked drop off (67-75%) after the first 1/4 inch, compared to a gradual drop off in the amines values as would be expected for a simple penetration of these compounds into the product. Highly significant ($p < 0.01$) correlations were found between NDBA, NDBzA, DBA and DBzA and depth slice. The finding confirmed that the nitrosamines are formed on the product surface.

A survey of commercial hams was carried out in cooperation with FSIS to determine to what extent nitrosamines are present in hams, and to obtain information on processing conditions that might favor nitrosamine formation. In the initial survey, 59 ham surface samples were obtained from 55 different processors and analyzed in duplicate. Of these, only 3 samples contained NDBA. One sample had 33.4 ppb NDBA with 4.0 ppb NDBzA, and 2 contained 3.8 and 4.2 ppb NDBA with 107.7 and 25.5 ppb NDBzA, respectively. Seven contained no detectable nitrosamines. However, of the remaining samples, 4.0 to 512.2 ppb NDBzA was detected, with 1/3 over 100 ppb.

Additional hams were obtained from the same processors whose samples yielded high results in the initial survey. The repeats tended to give variable results, but the results from 3 processors were consistently high; 512.2 and 746.9, 370.6 and 291.0, 486.8 and 550.3 ppb NDBzA. All of the samples containing over 100 ppb NDBzA were confirmed by mass spectrometry. The cotton thread used to wrap the rubber strips for the netting was found to contain from n.d. (<0.5 ppm) to 96.3 ppm nitrite (mean, 19.4 ppm), calculated as $NaNO_2$. In 6 samples containing high levels of nitrite, from 154.0 to 771.7 ppm nitrate (calculated as NaNO3) was detected. No relationship was found between the ham NDBzA surface values and nitrite or the more limited nitrate in the cotton. Because of the high degree of variability in NDBzA values, no correlation was found between the NDBzA surface values and any of the processing or smokehouse conditions. No correlation was found between the ham surface values and the NDBzA content of the unused nettings or

the netting values obtained after addition of nitrite (nitrosation potential).

The survey results showed the widespread use of nettings with reformulated rubber that produced NDBzA rather than NDBA, which was detected in earlier surveys. A number of producers were processing hams in stockings made from polyester thread; these hams had no detectable nitrosamines. All of the survey data obtained from the hams and nettings were supplied to FSIS. A study was conducted by us in which hams were processed in nettings previously found to give high levels of nitrosamine. The outer surface of the ham and a 1/4 in. thick center cut slice (n=33) were analyzed to determine if there was a correlation between the two nitrosamine values. Only hams having approximately the same diameter (5 1/4 in.) were used in this study in order to eliminate any effect due to differences in surface area. The NDBzA values for the outer surface covered a range from 11.5 to 805.1 ppb. A highly significant correlation ($p < 0.01$) between the outer and slice values was found. From the predictive equation developed, approximately 14% of the outer NDBzA level will be found in the slice. Or, expressing it differently, the surface to slice ratio for NDBzA was 7.1:1. This suggested that sampling the cross-sectional slice rather than the outer surface would be feasible. **IMPACT/TECH TRANSFER**: The incidence of NDBzA in netted hams is higher than previously encountered with NDBA. This data was given to FSIS for submission of a report to FDA.

OBJECTIVE D: Develop methods to reduce/eliminate nitrosamine formation.

PROGRESS D: Addition of nitrite to ham surface samples showed that it had the potential to form additional nitrosamine. This suggested that ingoing nitrite might be an important factor in determining how much NDBzA was formed. Hams were processed in nettings from the same batch; 12 were processed with 200 ppm and 12 with 100 ppm sodium nitrite. A highly significant reduction ($p < 0.01$) in NDBzA (31%) was noted in those made with the lower level of nitrite. While reduction of ingoing nitrite may be one way to reduce the nitrosamine content in netted hams, further investigations are needed before pursuing this approach, since nitrite exhibits a number of important properties in meat curing. Some ham producers leave the nettings on the hams for esthetic reasons. The hams can also be refrigerated for a long period of time prior to sale. Therefore, a study on the effect of netting contact time-NDBzA formation was carried out. In it, hams were stored with the nettings left on post-processing at refrigeration temperature (28 degs or 36 degs C) for either 6 or 12 weeks. Samples were taken for analysis at 2 week intervals. The overall results show that there are increasing levels of NDBzA with increasing netting contact time. This indicates that continued exposure of the ham to the netting results in increased NDBzA being present on the outer surface.

Analysis of the next 0.25 in. from the surface also showed that continued exposure of the ham to the netting results in increased NDBzA being present when compared to hams where the nettings were removed immediately after processing. These results strongly suggest that removal of the netting post-processing would help to reduce the amount of nitrosamine in the boneless hams processed in elastic rubber nettings.

Cooperative research was carried out with a rubber manufacturer to reformulate the rubber used for elastic nettings that would eliminate or significantly reduce the nitrosamine content in hams processed in these nettings. The company was successful in producing rubber with the needed properties using a combination of secondary amine-containing zinc dithiocarbamate derivatives. Evaluation of the reformulated rubber for nitrosamine content indicated that it had promise for this application. The work was terminated when the nitrosamine program was redirected. **IMPACT/TECH TRANSFER**: Recommended that FSIS require processors remove the nettings post-processing to reduce

the potential for additional nitrosamine to be formed.

OBJECTIVE E: Develop SFE methodology for nitrosamines of regulatory interest.

PROGRESS E: Research on the development of a supercritical fluid method (SFE) for nitrosamines, especially N-nitrosodibenzylamine (NDBzA), in hams processed in elastic rubber nettings was completed. Twenty one samples from the outer surface, ranging from N.D. to 157.3 ppb NDBzA, were analyzed by this method and by a solid phase extraction (SPE) method. The results from the two methods were found to be statistically equivalent. The S.D. of the SFE method was 1.7 ppb NDBzA (C.V. 2.7%) compared to 2.2 ppb with a C.V of 3.5% for the SPE method. The use of SFE was also investigated for its ability to extract nitrosamines from fried bacon since it is still being monitored by FSIS. Eighteen fried bacon samples were analyzed for volatile nitrosamines by SFE and the results compared to three other methods currently used for bacon analysis: MOD, LTVD, and SPE. N- Nitrosopyrrolidine (NPYR) and N-nitrosodimethylamine (NDMA) were the only nitrosamines detected. Individual NPYR values ranged from 0.7 to 20.2 ppb for SFE, 0.6 to 18.8 ppb for SPE, 1.0 to 24.6 ppb fo2r MOD, and 0.8 to 32.1 ppb for LTVD.

Analysis of variance of the NPYR data showed a significant difference ($p<0.05$) between the SFE (mean, 4.9 ppb) and SPE (mean, 4.5 ppb) results, and significant differences between these methods and the MOD (mean, 5.9 ppb) and LTVD (mean, 5.8 ppb) methods, which were higher than the other two methods, but not significantly different from each other. Overall, SFE was superior to the other methods with the highest recoveries, best repeatability, rapidity of analysis and solvent sparing characteristics. Similar results were obtained for SFE in bacon drippings after comparison with distillation and SPE methods. It is estimated that 20 samples can be analyzed per day by the SFE method compared to a maximum of 8-10 for the SPE method. Considerably less dichloromethane (DCM) is also required for SFE, 17 mL, compared to 450-500 mL for the other nitrosamine methods. **IMPACT/TECH TRANSFER**: FSIS personnel were trained at ERRC on the use of SFE to extract nitrosamines from fried bacon. This allows them to monitor bacon for nitrosamines using a procedure that requires less than 20 mL solvent.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO ANALYSES OF FIRE-EXPOSED MEAT SURFACES

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OBJECTIVE A: Utilize supercritical fluid extraction to obtain volatile and semivolatile compounds from fire exposed meats.

PROGRESS A: A supercritical fluid extraction method has been developed using supercritical carbon dioxide to analyze and characterize volatile and semi-volatile compounds from vegetable oil and meat samples. The SFE method used a Suprex Prepmaster SFE unit which was interfaced with a Finnigan GC-MS Incos 50 system. Extraction conditions of 100 atm and 50 °C enabled extraction of the volatile components without concurrent extraction of the lipids. The extracted volatiles were directly transferred into a Varian GC through a heated tube; the flow rate of CO_2 was regulated by a fritted restricter which was inserted into the GC injector. This research was utilized to identify and measure contaminants from meat products that were exposed to a warehouse fire; these meat products were acquired from the FSIS Midwest Laboratory.

The major volatile compounds were lipid oxidation products, such as hexanal and nonanal. Other compounds that were identified were aromatic and polycyclic hydrocarbons which included naphthalene, toluene, benzene, and ethylbenzene. Volatile concentrations from fire-exposed meat products were compared to control samples to determine composition differences. Quantitative data indicated that the concentration of naphthalene was greater in fire-exposed meats than the control samples. **IMPACT/ TECH TRANSFER**: Direct supercritical extraction from the meat samples proved to be a rapid and reproducible method to assess contamination in commercial meat products.

OBJECTIVE B: Utilize supercritical fluid methods to analyze compounds formed in irradiated meats.

PROGRESS B: A project has been developed utilizing the direct SFE method to identify compounds that are present in irradiated meats. Test samples obtained from the University of Illinois Department of Meat Science were irradiated at 3 dose levels; i.e., ground beef, blended to possess 10% fat content (1.44; 2.85 and 6.93 kGy) and 30 % fat content (1.82, 3.47 and 8.79 kGy); and a ham sample prepared with 15 % fat level (1.52, 3.16 and 7.85 kGy). Hydrocarbons from the breakdown of the fatty acids were identified and found to increase with the level of exposure to radiation. The relationships of the fat content on volatile formation from the radiation and the effect of the radiation on the volatiles during storage of the meat are presently being studied.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO DETERMINATION OF FAT LEVELS IN FOOD PRODUCTS

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OBJECTIVE A: Conduct collaborative study to show equivalence of SFE-based method to NLEA and similar protocols for fat analysis.

PROGRESS A: All sample matrices have been prepared for use in a collaborative study involving the determination of fat in meat products according to NLEA protocols. A protocol has been written by F. Eller which will be utilized for the purposes of this study. It was decided that a peer validated study, according to AOAC recommendations, would be utilized to save both money and time. New meat samples were characterized for total, saturated and monounsaturated fat content at Medallion Laboratories using the procedure reported by House, et.al. These samples were also analyzed by a similar procedure at Lancaster Laboratories for comparison to the Medallion results. Lancaster Labs also ran cholesterol content on all the meat samples generated at the University of Illinois-Department of Meat Science to date.

OBJECTIVE B Apply SFE and SFC for the analysis of cholesterol in meat products.

PROGRESS B: A method is under development by Scott Taylor which will permit the SFE of cholesterol from meat matrices along with its separation from other lipid coextractives. Utilizing a Suprex Autoprep 44, extraction conditions have been optimized for the removal of cholesterol from several food matrices, however the high level of lipid coextractives necessitates that a sorbent based cleanup step be integrated into the extraction step. Several sorbent types were evaluated for their ability to retard the extraction of cholesterol relative to other lipophilic constituents, both within the extraction cell as well as in the collection trap of the Autoprep 44. A cyanobonded silica packing was found to be most selective for cholesterol and has been used to partially cleanup an extract for subsequent analysis using capillary supercritical fluid chromatography. Cholesterol recoveries from several food matrices have averaged over 75%, including the determination of cholesterol in commercial braunschweiger.

OBJECTIVE C: Develop an enzymatic based hydrolysis/transesterification procedure for use in NLEA fat analysis.

PROGRESS C: A method has been developed to allow simultaneous hydrolysis followed by transesterification of the hydrolyzed triglyceride moieties to fatty acid methyl esters (FAMES), for use in NLEA fat analysis. Utilizing research reported by Dr. Michael Jackson, working in our chemical synthesis program using supercritical fluid media, we have used the lipase derived from C. Antarctica to produce FAMES from fat extracted from small meat samples. Extraction conditions of 2500 psi and 50 °C using SC-CO_2, permit the hydrolysis/tranesterification to be achieved without deactivating the enzyme. Using a home-built "FAME synthesizer", constructed around commercial syringe pumps from Isco, Inc., we have determined total, saturated, and monounsaturated fat levels in over 15 different meat matrices. The result from the lipase-catalyzed reaction in SC-CO_2

agree very well with independent results determined by classical acid hydrolysis and solvent extraction methods using chemical derivatization to form the FAME derivatives.

Recently this method has been automated with the aid of a Hewlett Packard "bridge" system which permits the lipase reaction to be conducted inside a commercial SFE vessel, with subsequent collection of the FAMES in a GC autoinjector vial. The autoinjector vial is then robotically transferred to the sample carousel of a HP 5890 GC for traditional GC FAME analysis. This research to date by Janet Snyder shows considerable promise and is currently being pursued.

OBJECTIVE D: Refinement of the SFE-based method for NLEA fat analysis.

PROGRESS D: Some additional studies have been performed to better integrate SFE into an established NLEA fat protocol. Dr. Fred Eller has performed studies to better understand the role of using CO_2 cylinders pressurized with He gas on the recorded drop in solubility for fats/oils in SC-CO_2. Our studies indicate that the presence of helium can reduce the triglyceride content of SC-CO_2 between 33-50% by weight, depending on the extraction pressure and temperature. Obviously, this effect must be taken into account when using instrumentation that requires He-headspace carbon dioxide to function properly.

IMPACT/TECH TRANSFER: Dr. Eller has also performed fat extraction studies on different commercial instruments (Hewlett Packard 7680, Isco SFX-2-10, Suprex Autoprep 44) and shown that equivalent results with respect to NLEA fat analysis can be obtained on any instrument within the limits of experimental error. These instruments were recently demonstrated to visitors from FSIS' Eastern Laboratory who spent a week at NCAUR reviewing our SFE-based fat extraction techniques. They will participate in the above-mentioned peer validated study later this fall.

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REFERENCE MATERIALS FOR NUTRITIONAL ANALYSIS

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OBJECTIVE A: Development and use of highly accurate methodology required to provide reference materials (RM).

PROGRESS A: In collaboration with scientists from the National Institute of Standards and Technology, a method has been developed to determine fatty acids in a composite food RM using isotope dilution mass spectrometry and has been used to characterize a new diet composite Standard Reference Material (SRM) .

IMPACT/TECH TRANSFER: This will provide a critically needed SRM for individual fatty acids in food materials for use in nutritional labeling regulations analysis by both FSIS and the food industry.

OBJECTIVE B: Research necessary to choose representative food matrix candidate reference materials

PROGRESS B: A scheme has been proposed to identify foods as chemical matrices representative of a range of foods as candidate reference materials This scheme defines a food matrix by its position in the food triangle with each point defined as representing 100% and the opposite side 0% of the normalized content of the three major components of foods, Fat, Carbohydrate, and Protein.

IMPACT/TECH TRANSFER: This scheme is being considered for adoption by AOAC INTERNATIONAL to choose matrices representative of all foods for collaborative studies and method validation.

OBJECTIVE C: Define and establish of a national infrastructure to provide RM.

PROGRESS C: Proposals were developed in collaboration with FSIS and ARS to develop two new SRM, a Canned Meat Material, and a Mixed Diet Material and have been forwarded to NIST for consideration as FY96 development projects for new SRM's. Plans are being drafted for a proposal to"Accelerate Development of Reference Materials for Food/Nutrition," through the Working Group on Reference Materials, Interagency Committee of Human Nutrition Research. Activity continues to establish and develop the Technical Division on Reference Materials, AOAC INTERNATIONAL to facilitate availability and use of RM in method validation, implementation and use of "Official Methods of Analysis".

IMPACT/TECH TRANSFER: These activities will continue progress to establish the necessary national infrastructure required to improve food/nutrition measurements.

PUBLICATIONS:

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NIR SPECTROMETRY TO MEASURE NUTRIENTS

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Termination Date:
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OBJECTIVE A: To study the usefulness on NIR technology in nutrient analysis of meat and poultry products for nutrition labeling and other regulatory samples and assess its ability to reduce the amount of hazardous waste from current laboratory methods.

PROGRESS A: Classification of prior temperature history of chicken breasts The labelling of raw poultry products as 'fresh' has been used historically to describe products that have not been previously frozen to -18 °C. Recently, the Food Safety Inspection Service has proposed to prohibit the use of the term 'fresh' on the labeling of raw poultry products whose temperature has ever been below -3 °C. In this work, spectroscopic discriminant models were developed to classify the temperature to which poultry had been chilled. Deboned breast fillets were chilled to simulate the time between processing and point of distribution by the retailer. Test conditions included five holding chamber temperatures (4,0,-3,-12,-18 °C). Samples were scanned from 400 to 2500 nm immediately after deboning, and then placed in one five chambers. After storage, samples from 0,-3,-12, and -18 °C were allowed to temper to 4 °C and rescanned. Spectra of tempered breasts were used in discriminant analysis for classification of samples into the 5 and 3 temperature classes. Within a storage class, no samples were uniquely classified as belonging to one of the 5 class models. If one designates the -18 and -12 °C samples as 'frozen' and the -3,0, and 4 °C as 'unfrozen', the 3 class model classified 75% and 85% of the samples correctly. Classification of prior temperature history appears feasible with NIR. However, the classification models in their current state are not accurate enough for regulatory purposes.

The above project dominated the year, however the FSIS projects on meats for fat, protein and moisture are continuing. Collaborations with Golden States Foods give us all the hamburger samples we need to update and validate the calibration. We need sausage samples from FSIS to augment the data set and proceed to the next step of the project.

PUBLICATIONS:

Windham, W. R., F. E. Barton, II, B. G. Lyon, and C. E. Lyon. 1995. Classification of priior temperature history of chilled chicken breasts by NIR. Proc. 7th Intern. Conf. on Near Infrared Spectroscopy. Montreal, Canada, (In Press).

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ARS FOOD SAFETY RESEARCH ADDRESSING FSIS NEEDS

Subject Area and Laboratory Location Index

II. Pathogen control during slaughtering and processing

III. Post-slaughter pathogen control

IV. Residue detection and nutrient analysis

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